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(54) Title: tRNA BINDING-DEPENDENT INHIBITION OF MICROBIAL PATHOGEN GROWTH


(57) Abstract

The present invention relates to a method of specifically inhibiting growth of microbes, such as bacteria, fungi, or viruses, and to compositions useful in the present method. According to the method, specific tRNA-dependent inhibition of the growth of microbial pathogens can be achieved through use of tRNA binding molecules. For example, a tRNA binding molecule, such as a mutant aminoacyl-tRNA synthetase, which is capable of binding tRNA, but incapable of aminoacylation, can be selectively toxic to a selected microbial pathogen, leading to inhibition of (i.e., a reduction in or arrest of) growth of the pathogen, while sparing the host cell.

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# tRNA BINDING-DEPENDENT INHIBITION OF MICROBIAL PATHOGEN GROWTH

## Description

### Background

Antibiotics are chemical substances produced by various microorganisms, including bacteria, fungi, and actinomycetes, which suppress the growth of other microorganisms and, in some cases, eventually destroy those microorganisms. The term antibiotic is also used to refer to synthetic antibacterial agents which are not products of microbes, such as sulfonamides and quinolones. Since the introduction of penicillin, antimicrobial therapy has become routine. For example, today, at least 30% of all hospitalized patients receive one or more courses of antibiotic therapy (Sande, M.A. et al., 1990, In: Goodman and Gilman's The Pharmacological Basis of Therapeutics, Eighth edition, A.G. Gilman et al., Eds., (Pergamon Press: New York), pp. 1018-1046). Although advances in understanding the molecular mechanisms of bacterial, fungal and viral growth and replication have led to the rational development of antimicrobial agents useful in the chemotherapy of microbial diseases, the widespread use of antimicrobial agents over the past 50 years has led to the emergence of resistant pathogens (Amábile-Cuevas, C.F. and M.E. Chicurel, Cell, 70:189-199 (1992)). The rapid evolution of microbial resistance to antimicrobial agents, such as the resistance of bacteria to antibiotics, has created a continuing need for new drug targets and new drugs. Apart from the development of

microbial resistance, new strategies of antimicrobial therapy and antimicrobial agents, which would be generally applicable to a variety of microbial pathogens, yet which would minimize side effects in the host, are needed.

Summary of the Invention

The present invention relates to a method of specifically inhibiting growth of microbes, such as bacteria, fungi, or viruses. According to the method, specific tRNA-dependent inhibition of the growth of microbial pathogens can be achieved through use of tRNA binding molecules. For example, a tRNA binding molecule, such as a mutant aminoacyl-tRNA synthetase, capable of binding tRNA, but incapable of aminoacylation, can be selectively toxic to a selected microbial pathogen, leading to inhibition of (i.e., a reduction in or arrest of) growth of the pathogen, while sparing the host cell. The activity of tRNA binding molecules, such as mutant aminoacyl-tRNA synthetases, is tRNA-binding dependent and may result from the ability of these molecules to sequester specific tRNA, thereby rendering the tRNA unavailable to the translational apparatus. The ability of specific tRNA-binding molecules to sequester microbial tRNA selectively can lead to inhibition of microbial protein synthesis, leading to inhibition of microbial growth (i.e., a reduction in or arrest of growth or death), with minimal effect on the host.

The invention further relates to tRNA binding molecules useful in the method, such as a mutant aminoacyl-tRNA synthetase capable of binding tRNA, a tRNA binding fragment of an aminoacyl-tRNA synthetase, or a tRNA-binding synthetase mimetic. By virtue of their preferential action against specific microbial tRNA as compared with host tRNA, the tRNA-binding molecules of the present invention specifically inhibit microbial protein

synthesis, thereby specifically inhibiting microbial growth. Thus, the method of the present invention provides a new strategy of antimicrobial therapy useful against a large variety of pathogens, and the tRNA binding molecules represent a new class of antimicrobial agents useful in antimicrobial therapy or gene therapy of microbial infection, capable of selective inhibition of growth of pathogens and limited toxicity to the host.

Brief Description of the Drawings

Figure 1 is an illustration of the secondary structure of isoleucyl-tRNA synthetase (IleRS) and of portions of the primary sequences of known IleRS and MetRS proteins. The structure of IleRS is based on sequence alignments with the three dimensional structure of *E. coli* MetRS (Starzyk, R. et al., Science, 237:1614-1618 (1987); Brunie, S. et al., J. Mol. Biol. 216(2):411-424 (1990)). The N-terminal nucleotide binding fold, C-terminal helical domain, and anticodon binding regions are labeled. CP1 and CP2 refer to connective polypeptide insertions 1 and 2 in the nucleotide binding fold. The rectangles indicate α-helices, and the pentagons indicate β-sheets. The sequence surrounding the mutations introduced into IleRS, and the corresponding sequences for four isoleucyl- and five methionyl-tRNA synthetases are shown (see also SEQ ID NO:2 through SEQ ID NO:9 and SEQ ID NO:11 through SEQ ID NO:18). Residues inside shaded boxes are conserved between *Escherichia coli* MetRS and the other synthetases. The sequences illustrated are *Escherichia coli* IleRS (Ec-I) (SEQ ID NO:1 and SEQ ID NO:10; Webster, T.A. et al., Science, 226:1315-1317 (1984)), *Saccharomyces cerevisiae* IleRS (Sc-I) (SEQ ID NO:2 and SEQ ID NO:11; Englisch, U. et al., Biol. Chem. Hoppe-Seyler, 368:971-979 (1987)), *Methanobacterium thermoautrophicum* IleRS (Mt-I) (SEQ ID NO:3 and SEQ ID NO:12; Jenal, U. et al., J. Biol. Chem.,

266(16):10570-10577 (1991)), *Tetrahymena thermophila* IleRS (Tet-I)(SEQ ID NO:4 and SEQ ID NO:13; Csank, C. and D.W. Martindale, J. Biol. Chem., 267(7):4592-4599 (1992)), *Thermus thermophilus* MetRS (Tmt-M)(SEQ ID NO:5 and SEQ ID NO:14; Nureki, O. et al., J. Biol. Chem., 266(5):3268-3277 (1991)), *Saccharomyces cerevisiae* mitochondrial MetRS (Scm-M)(SEQ ID NO:6 and SEQ ID NO:15; Tzagoloff, A. et al., Eur. J. Biochem., 179:365-371 (1989)), Saccharomyces cerevisiae MetRS (Sc-M)(SEQ ID NO:7 and SEQ ID NO:16; Walter, P. et al., Proc. Natl. Acad. Sci. USA, 80:2437-2441 (1983)), *Bacillus stearothermophilus* MetRS (Bst-M)(SEQ ID NO:8 and SEQ ID NO:17; Mechulam, Y. et al., Nucleic Acids Res., 19(13):3673-3681 (1991)), and *Escherichia coli* MetRS (Ec-M)(SEQ ID NO:9 and SEQ ID NO:18; Dardel, F., J. Bacteriol., 160(3):1115-1122 (1984)).

Figures 2A-C are illustrations of the growth rates of cultures of *E. coli* MV1184, harboring plasmids encoding wild-type or mutant IleRS genes. The cell densities ($OD_{600}$) of cultures of *E. coli* MV1184 carrying wild-type (Figure 2A), D96A mutant (Figure 2B) or D96A/K732T mutant (Figure 2C) IleRS genes, grown under induced or uninduced conditions, are plotted as a function of time. Bold arrowheads indicate the time of addition of IPTG.

Figure 3 is a graph illustrating the in vitro aminoacylation activity of the D96A IleRS mutant. The graph shows the extent of aminoacylation of purified *E. coli* $tRNA^{Ile}$ by wild-type IleRS, D96A IleRS or the mutant IleRS purified from the MI1 strain. No activity above background could be detected for either the D96A mutant or the MI1 mutant under the conditions of the assay.

Figures 4A-B are illustrations of the inhibition of wild-type *E. coli* IleRS activity by mutant *E. coli* IleRS in vitro. Wild-type aminoacylation activity was measured

under conditions of increasing mutant protein concentration (O, no added protein; ▲, 0.1 μM mutant protein; ■, 0.5 μM mutant protein; ●, 1.0 μM mutant protein). Figure 4A illustrates the effect of the D96A mutant and Figure 4B illustrates the effect of the D96A/K732T double mutant.

Figure 5 is an illustration of the effect of *E. coli* D96A or D96A/K732T mutant IleRS proteins on the extent of aminoacylation of crude bovine tRNA by a human HeLa cell extract in vitro. Aminoacylation activity of the HeLa cell extract was measured by the incorporation of radioactive isoleucine into charged tRNA under the following conditions: no added inhibitor protein (●), 1.0 μM D96A IleRS (■), or in the presence of 1.0 μM D96A/K732T IleRS (▲).

Figure 6 is an illustration of the growth rates of cultures of *E. coli* MV1184, harboring plasmids encoding mutant IleRS genes. The cell densities ($OD_{600}$) of cultures of *E. coli* MV1184 carrying G56A mutant or F570S mutant IleRS genes, grown under uninduced or induced (+ IPTG) conditions, are plotted as a function of time. Induction with IPTG was carried out at time = 3.0 hours.

## Detailed Description of the Invention

The aminoacylation of transfer RNA (tRNA) is catalyzed by enzymes of the aminoacyl-tRNA synthetase family. The fidelity of translation depends upon the incorporation of the correct amino acid in a growing polypeptide chain in response to each codon of the mRNA. Fidelity of translation is determined in part by the correct base pairing of the anticodon of each aminoacylated tRNA with the complementary codon in the mRNA. In addition, the fidelity of translation depends upon the attachment of the proper amino acid to each tRNA,

a reaction which is catalyzed by aminoacyl-tRNA synthetases specific for each amino acid. There is generally one synthetase for each amino acid; however, because of the degeneracy of the genetic code, there may be several tRNA isoacceptors for each amino acid. The aminoacyl-tRNA synthetase for a selected amino acid is capable of aminoacylating or "charging" each of the isoacceptor tRNAs for that amino acid (i.e., cognate tRNAs).

The aminoacyl-tRNA synthetases catalyze the esterification of an amino acid to cognate tRNA in a two-step reaction. In the first step of the reaction, the amino acid is activated by condensation with ATP to form an enzyme-bound aminoacyl-adenylate. In the second step of the reaction, the amino acid is joined via an ester linkage to the 2′- or 3′-hydroxyl group at the 3′ end of a cognate tRNA molecule. The overall reaction can be expressed as follows, where $AA_i$ is a selected amino acid, $tRNA^i$ is a cognate tRNA, and $PP_i$ is pyrophosphate:

$$AA_i + ATP + tRNA^i \rightarrow AA_i\text{-}tRNA^i + AMP + PP_i$$

In vivo, the aminoacylated or charged tRNA is rapidly bound by translation factors and is subsequently delivered to the ribosomes.

Because of the central role of aminoacyl-tRNA synthetases and tRNAs as interpreters of the genetic code in the accurate translation of proteins, alterations in the efficiency or accuracy of aminoacylation reactions in vivo can be detrimental to cells. For example, previous studies have shown that a mutant *E. coli* glutaminyl-tRNA synthetase capable of misacylating tRNA reduced the growth rate of *E. coli* cells (Inokuchi, H. et al., Proc. Natl. Acad. Sci. USA, 81:5076-5080 (1984)). Thus, defects in

the accuracy of aminoacylation can be detrimental to growth. As shown herein, according to the method of the present invention, specific tRNA-dependent inhibition of the growth of microbial pathogens can be achieved through use of tRNA binding molecules which appear to influence the efficiency of aminoacylation. In particular, tRNA synthetase-induced growth arrest has been achieved through the introduction of a mutant aminoacyl-tRNA synthetase in a microbial cell, indicating that essential functions mediated by aminoacyl-tRNA synthetases and tRNAs provide suitable targets for antimicrobial agents.

According to the present method, a tRNA binding molecule, such as a mutant aminoacyl-tRNA synthetase, is constructed and introduced into a microbial cell. When introduced into a selected microbial cell or expressed in a selected microbial cell in sufficient amounts, the tRNA binding molecule is capable of binding a specific microbial tRNA, and can be selectively toxic to the microbial pathogen, leading to inhibition of (i.e., a reduction in or arrest of) growth of the pathogen, while sparing the host cell.

The toxicity of tRNA binding molecules, such as mutant aminoacyl-tRNA synthetases, may result from the ability of these molecules to sequester specific tRNA, thereby rendering the tRNA unavailable to the translational apparatus. This inhibition of microbial protein synthesis results in an inhibition of microbial growth. Different tRNA binding molecules may have different inhibitory effects on microbial growth, including reduction of microbial growth or arrest of microbial growth, and/or induction of death.

According to the present method, selective inhibition of microbial growth is achieved, such that antimicrobial effect can be observed while toxicity of the tRNA binding molecule to host cells is minimized. Specific tRNA-

binding molecules are designed or selected to sequester microbial tRNA selectively with minimal effects on host cell tRNA. By virtue of their specificity of action against specific microbial tRNA as compared with host tRNA, the tRNA-binding molecules of the present invention specifically inhibit microbial protein synthesis, thereby specifically inhibiting microbial growth. Active or inactive aminoacyl tRNA-synthetase molecules may have the requisite tRNA binding capability.

The invention further relates to tRNA binding molecules useful in the method, such as a mutant aminoacyl-tRNA synthetase capable of binding tRNA, a tRNA binding fragment thereof, or a tRNA-binding synthetase mimetic (e.g., a peptide analog of tRNA synthetase). As shown in Example 1, a mutant *E. coli* aminoacyl-tRNA synthetase which is defective in aminoacylation, but is capable of binding tRNA, is toxic to *E. coli* cells. These results demonstrate that toxicity can be achieved in the absence of measurable aminoacylation activity. Thus, tRNA binding molecules useful in the present invention need not possess aminoacylation activity.

When a mutant aminoacyl-tRNA synthetase is selected as the tRNA binding molecule, an aminoacyl-tRNA synthetase defective in aminoacylation is preferred, because retention of <u>in vivo</u> aminoacylation activity by a mutant aminoacyl-tRNA synthetase may increase the concentration of mutant protein required for effective inhibition of microbial growth. Moreover, alterations which relax the specificity of aminoacylation or tRNA binding may result in a molecule with activity detrimental to host cells. Thus, a mutant aminoacyl-tRNA synthetase which retains specificity of binding for one or more cognate tRNAs (i.e., does not significantly bind or aminoacylate non-cognate tRNAs) is preferred.

A variety of inactive aminoacyl-tRNA synthetase mutants can be used in the present method, provided they retain ability to bind tRNA. For example, an inactive aminoacyl-tRNA synthetase having a defect in amino acid binding, ATP binding, aminoacyl-adenylate formation or transfer of amino acid to tRNA, or a combination of defects, is useful in the method (see e.g., Example 1 and Example 3). Mutant aminoacyl-tRNA synthetases capable of specific binding of tRNA with increased affinity may be particularly effective; however, as shown herein, a mutant isoleucyl-tRNA synthetase having a dissociation constant quite similar to wild-type isoleucyl-tRNA synthetase (0.33 μM for wild-type and 0.48 μM for D96A mutant) displayed antimicrobial activity. Although aminoacyl-tRNA synthetases normally bind to all isoaccepting tRNAs in the cell, effective inhibition of protein synthesis could be accomplished by sequestering a single tRNA isoacceptor.

A mutant aminoacyl-tRNA synthetase can be derived from an aminoacyl-tRNA synthetase from the same microbial organism (i.e., a homologous source) or from a different microbial organism (i.e., a non-homologous source). For example, an aminoacyl-tRNA synthetase obtained from a selected microbial cell (e.g., *E. coli*) can be mutated and used in the same type of microbial cell (i.e., *E. coli*) to inhibit growth. In this case, the aminoacyl-tRNA synthetase mutant is used in a homologous system and is said to be a homologous enzyme. Alternatively, an aminoacyl-tRNA synthetase obtained from a selected microbial cell (e.g., *E. coli*) can be mutated and used in a different type of microbial cell (e.g., *Shigella*) to inhibit growth. In the latter case, the aminoacyl-tRNA synthetase mutant is used in a heterologous system and is said to be a heterologous enzyme.

Mutant aminoacyl-tRNA synthetases can be obtained via mutagenesis of cloned aminoacyl-tRNA synthetase genes. A

variety of mutagenesis strategies are possible, which may generate one or more point mutations, deletions, insertions, or truncations or combinations thereof. For example, suitable mutants can be generated by random mutagenesis (e.g., chemically induced) and screening for the desired properties. Alternatively, suitable mutants can be obtained by random mutagenesis of a selected region, as, for instance, by cassette mutagenesis (see e.g., Clarke, N.D. et al., Science, 240:521-523 (1988)). Because the structure and function of aminoacyl-tRNA synthetases has been investigated extensively, site-directed mutagenesis can be used to obtain mutant enzymes having a specific alteration at a selected position or within a particular region selected for its known or suspected role in a particular function (see e.g., Example 3, Burbaum, J.J. and P. Schimmel, J. Biol. Chem., 266(26): 16965-16968 (1991); Cusack, S. et al., Nucleic Acids Res., 19: 3489 (1991); Moras, D., Trends in Biochem. Sci., 17: 159 (1992); Schimmel, P., Ann. Rev. Biochem., 56: 125-158 (1987), and references cited therein).

The properties of a particular mutant can be characterized by a variety of methods, including in vivo complementation assays (see e.g., Examples 1 and 3), or enzymatic assay (see e.g., Examples 1-3). The two steps of the aminoacylation reaction can be characterized; adenylate formation can be monitored in an assay of pyrophosphate exchange and aminoacylation can be monitored by incorporation of amino acid into charged tRNA. Inactive mutants do not display activity in assays of in vitro aminoacylation activity or display little or no activity in complementation assays in vivo. Further biochemical analysis of the kinetics of amino acid, ATP and tRNA binding can be conducted.

To determine whether a mutant aminoacyl-tRNA synthetase is toxic to a microbial cell, the synthetase is

introduced into the microbial cell and its effect on cell growth in monitored by standard methods (e.g., in culture). For instance, nucleic acid encoding the mutant protein can be inserted into an expression vector, and the vector can be introduced into a microbial cell in which the protein is expressed. Suitable expression vectors are available for a variety of microbial systems, including bacterial, mycobacterial, and fungal vectors. The effect of expression of a mutant synthetase as compared with an appropriate control (e.g., a vector control, or vector carrying wild-type synthetase) can be monitored in the target microbial cell. In addition, derivatives of a selected mutant which contain one or more additional mutations in tRNA binding can be constructed, and the activity of such mutants can be compared with the original selected mutant to demonstrate the tRNA binding-dependence of inhibition (see e.g., Example 1).

In one embodiment, the mutant aminoacyl-tRNA synthetase is expressed from an inducible promoter. Upon induction, the mutant synthetase is expressed in the cell. Inducible expression vectors for expression of genes in microbial systems are available. Mutant synthetases which are toxic to the microbial cell will, upon expression, cause inhibition of growth, observable as a reduction in growth rate or arrest of growth or cell death. Microbial cells susceptible to tRNA-binding molecule toxicity are suitable target pathogens.

In addition, the toxic activity of a mutant tRNA synthetase protein can be further characterized in vitro. An extract containing the mutant tRNA synthetase is obtained. The mutant enzyme may optionally be purified as needed. The inhibitory activity of the mutant protein upon aminoacylation catalyzed by microbial synthetases can be monitored. Microbes which are candidate target pathogens, and whose growth may be inhibited according to

the method of the present invention, can be identified by the ability of a mutant aminoacyl-tRNA synthetase to inhibit the extent of aminoacylation catalyzed by a microbial aminoacyl-tRNA synthetase or microbial cell extract containing aminoacyl-tRNA synthetases.

The ability of a tRNA binding molecule, such as a mutant tRNA-synthetase to selectively inhibit microbial growth, but to spare the infected host can be assessed in vitro or in vivo. For instance, the ability of a mutant aminoacyl-tRNA synthetase to inhibit the extent of aminoacylation catalyzed by a host aminoacyl-tRNA synthetase or cell extract containing aminoacyl-tRNA synthetases can be assessed. Similarly, the mutant aminoacyl-tRNA synthetase can be introduced into cultured host cells and assessed for toxicity. In one embodiment, nucleic acid encoding a selected mutant aminoacyl-tRNA synthetase is introduced into a host cell by means of an expression vector, and toxicity of the mutant protein is assessed. Preferable tRNA binding molecules will have minimal effects on aminoacylation by host synthetases and host cell growth. Specificity of action against microbial pathogens, while sparing the host cell, due to the selectivity and specificity of binding interactions and aminoacylation activity of the selected aminoacyl-tRNA synthetase, can thereby be achieved according to the method.

A fragment of mutant aminoacyl-tRNA synthetase, which retains tRNA binding function, or a fragment of a wild-type aminoacyl-tRNA synthetase which is inactive but retains ability to bind tRNA, can also be used in the method. Suitable fragments of mutant synthetases can be identified by their toxic effect, using the in vitro or in vivo assays described above. The fragments can be obtained by digestion of mutant proteins or by recombinant methods via the expression of a portion of an aminoacyl-

tRNA synthetase (e.g., and N- or C-terminal fragment or derivative containing an internal deletion).

Production of a Toxic tRNA Binding Molecule

In one embodiment, site-directed mutagenesis was used to create an inactive, but toxic, *E. coli* aminoacyl-tRNA synthetase. In *E. coli*, the aminoacyl-tRNA synthetases are essential enzymes. Based on conserved sequence and structural motifs, the twenty synthetases have been divided into two classes of ten enzymes each (see Table 1, below; Eriani G. et al., Nature, 347:203-206 (1990); Ludmerer S.W. et al., J. Biol. Chem., 262(22):10801-10806 (1987); Webster T.A. et al., Science, 226:1315-1317 (1984)). Class I enzymes have a well conserved N-terminal nucleotide binding fold (Rossmann fold) responsible for amino acid binding, aminoacyl-adenylate formation and tRNA acceptor helix docking, joined to a less conserved C-terminal domain responsible for tRNA anticodon loop binding (Burbaum J.J. and P. Schimmel, Biochemistry, 30(2):319-324 (1991); Shepard A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992); Shiba K. and P. Schimmel et al., Proc. Natl. Acad. Sci. USA, 89:1880-1884 (1992); Brunie, S. et al., J. Mol. Biol., 216:411-424 (1990); Rould, M.A. et al., Science, 246:1135-1142 (1989)).

Based upon additional sequence similarities, a subgroup containing the five most closely related class I synthetases has been defined. This subgroup includes the synthetases specific for cysteine, isoleucine, leucine, methionine, and valine (Hou, Y-M. et al., Proc. Natl. Acad. Sci. USA, 88:976-980 (1991)). The N- and C-terminal domains of this subgroup of synthetases appear to act independently. Mutations in the N-terminal domain which almost completely abolish amino acid binding or aminoacyl-adenylate formation do not affect tRNA binding activity of

Table 1

| CLASS I ENZYMES | CLASS II ENZYMES |
|---|---|
| Arg-tRNA synthetase | Ala-tRNA synthetase |
| Cys-tRNA synthetase | Asn-tRNA synthetase |
| Glu-tRNA synthetase | Asp-tRNA synthetase |
| Gln-tRNA synthetase | Gly-tRNA synthetase |
| Ile-tRNA synthetase | His-tRNA synthetase |
| Leu-tRNA synthetase | Lys-tRNA synthetase |
| Met-tRNA synthetase | Phe-tRNA synthetase |
| Tyr-tRNA synthetase | Pro-tRNA synthetase |
| Trp-tRNA synthetase | Ser-tRNA synthetase |
| Val-tRNA synthetase | Thr-tRNA synthetase |

the C-terminal domain (Clarke N.D. et al., Science, 240:521-523 (1988); Ghosh G. et al., Biochemistry, 30:9569-9575 (1991)). Similarly, C-terminal mutations affecting tRNA binding have little effect on amino acid binding, aminoacyl-adenylate formation, or aminoacylation of a microhelix based on the tRNA acceptor stem (Ghosh G. et al., Biochemistry, 29(9):2220-2225 (1990); Kim, S. and P. Schimmel, J. Biol. Chem., 267:15563-15567 (1992); Shepard, A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992)). Further evidence for the modularity of structure of members of this subgroup of class I synthetases comes from demonstrations of non-covalent assembly of active enzymes from discrete pieces (Burbaum, J.J. and P. Schimmel, Biochemistry, 30(2):319-324 (1991); Shiba K., and P. Schimmel, Proc. Natl. Acad. Sci. USA, 89:1880-1884 (1992); Shiba K. and P. Schimmel, J. Biol. Chem., 267:22703-22706 (1992)). The finding that mutations which affect one interaction or activity (e.g., binding to the anticodon) need not affect another function

(e.g., amino acid activation and acceptor helix interactions), permits the construction of inactive mutants which retain tRNA binding activity. Therefore, isoleucyl-tRNA synthetase was selected for mutagenesis.

Figure 1 illustrates the predicted secondary structure of the N-terminal domain of *E. coli* isoleucyl-tRNA synthetase. This representation is based on sequence alignments with the *E. coli* methionyl-tRNA synthetase; the three-dimensional structure of *E. coli* methionyl-tRNA synthetase has been solved (Brunie, S. et al., J. Mol. Graphics, 5(1):18-21 (1987); Brunie, S. et al., J. Mol. Biol., 216(2):411-424 (1990)). The structure is comprised of alternating $\beta$-strands and $\alpha$-helices arranged in a Rossmann nucleotide binding fold. A large and variable insertion designated CP1 (connective polypeptide 1) occurs between the third $\beta$-strand ($\beta_C$) and $\alpha$-helix ($\alpha_C$) and a second insertion CP2 occurs between the fourth $\beta$-stand ($\beta_D$) and $\alpha$-helix ($\alpha_D$). Also shown are aligned sequences of the known synthetases specific for isoleucine and methionine. The high degree of N-terminal sequence homology between the subgroup of class I synthetases has made possible alignments of conserved and potentially critical residues (Ghosh, G. et al., Biochemistry, 30:9569-9575 (1991); Hou, Y-M. et al., Proc. Natl. Acad. Sci. USA, 88:976-980 (1991); Shepard, A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992); Shiba, K. and P. Schimmel, Proc. Natl. Acad. Sci. USA, 89:1880-1884 (1992)).

An Asp52→Ala mutation in the N-terminal domain of *E. coli* methionyl-tRNA synthetase results in a stable protein characterized by a greatly reduced $k_{cat}$ for aminoacyl-adenylate formation (Ghosh, G. et al., Biochemistry, 30:9569-9575 (1991)). An Asp→Ala mutation was introduced into the analogous location (i.e., at position 96) in the

nucleotide-binding fold of the class I *E. coli* isoleucyl-tRNA synthetase (IleRS) (See Figure 1, SEQ ID NO:1; see also, the DNA sequence of *E. coli* IleRS, SEQ ID NO:19, and predicted protein sequence, SEQ ID NO:20, as reported by Webster, T.A. *et al.*, Science, 226:1315-1317 (1984); the DNA and predicted protein sequence of *E. coli* IleRS (isoleucyl-tRNA ligase, *ileS*), SEQ ID NO:21 and SEQ ID NO:22, as reported by Yura *et al.*, Nucl. Acids Research, 20(13): 3305-3308 (1992) and entered on the Entrez Database, National Center of Biotechnology Information, NIH, 8600 Rockville Pike, Bethesda, MD 20894, (301) 496-2475), Accession No. D10483; and Dessen, P. *et al.*, 1993, "Sequence verification of *E. coli* isoleucyl-tRNA synthetase by matrix assisted laser desorption mass spectrometry", 15th International tRNA Workshop, Book of Abstracts, No. F.56, regarding solving nine of the sequence differences between these sequences and reporting that the protein is 939 amino acids in length). (Unless indicated otherwise, reference to specific nucleotide positions or amino acid residues in the subject application is with reference to SEQ ID NO:19 and SEQ ID NO:20, respectively.) The resulting mutant was characterized in an in vivo complementation assay, and by in vitro assay. As shown in Example 1, the mutant protein, referred to herein as D96A IleRS, was stable, yet devoid of activity, as measured by its inability to complement an *ileS* null tester strain (IQ844/pRMS711). Furthermore, although the Asp96→Ala replacement resulted in inactivation of enzymatic activity, it did not disrupt the ability of the protein to bind isoleucine tRNA specifically.

The mutant D96A IleRS gene was inserted into an inducible expression vector for introduction into *E. coli*. The vector was introduced into in *E. coli* containing a wild-type *ileS* chromosomal allele. Induction of

expression of the inactive D96A mutant revealed a strong toxic effect (Example 1, Figure 2B).

Introduction of a second Lys732→Thr substitution, previously shown to weaken tRNA binding, yielded an inactive Asp96→Ala/Lys732→Thr double mutant. Expression of the double mutant was not lethal to *E. coli*, suggesting that tRNA binding is required for the inhibitory effect of the original D96A mutant (Example 1, Figure 2C). Consistent with these observations, the D96A mutant, but not the double mutant, significantly inhibited in vitro charging (i.e., aminoacylation) of isoleucine tRNA by the wild-type enzyme (Example 1, Figures 4A and 4B). Additional studies revealed that neither the Asp96→Ala mutant, nor the double mutant, significantly affected the aminoacylation activity of mammalian cell extracts as determined by in vitro aminoacylation of crude mammalian tRNA with isoleucine (Example 2, Figure 5).

These results demonstrate a dominant lethal phenotype upon induction of a catalytically inactive mutant tRNA synthetase in its homologous organism. The data strongly suggest that a tRNA binding-dependent activity is responsible for toxicity of the D96A mutant. Because specific tRNAs are not present in substantially greater concentrations than their cognate synthetases (Jakubowski, H. and E. Goldman, J. Bacteriology, 158(3):769-776 (1984)), an inactive enzyme which retained its tRNA binding activity could, in present in sufficient concentration, sequester much of its cognate tRNA and thereby inhibit protein synthesis partially or completely by effectively starving the cells for a specific charged tRNA species. Thus, through a tRNA-binding dependent reduction in the pool of a specific charged tRNA, inhibition of microbial growth is possible. Starvation for a particular charged tRNA species may lead to toxicity and inhibition of growth via secondary effects on specific

essential proteins or via a general effect on protein synthesis.

One advantageous aspect of using a tRNA binding molecule such as an aminoacyl-tRNA synthetase mutant capable of binding charged or uncharged tRNA, is that tRNA may be captured and sequestered prior to aminoacylation by the endogenous synthetase or subsequent to aminoacylation. An alternative or additional contribution to toxicity is the tRNA-binding dependent deacylation or "editing" activity of IleRS (Schreier, A.A. and P.R. Schimmel, Biochemistry, 11:1582-1589 (1972)). This hydrolytic activity causes a slow deacylation of charged $tRNA^{Ile}$ in the absence of the products (AMP and $PP_i$) of the aminoacylation reaction. The hydrolytic activity is apparently independent of the aminoacylation function. In separate experiments, the capacity of the D96A mutant to deacylate Ile-$tRNA^{Ile}$ was demonstrated. Deacylation of the non-binding D96A/K732T mutant was reduced at least 75%, presumably because of a lack of tRNA binding. Expression of an aminoacylation-deficient IleRS capable of deacylating tRNA could reduce cellular levels of aminoacylated $tRNA^{Ile}$ available for protein synthesis. By overcoming the synthetic capability of the endogenous wild-type enzyme, deacylation activity may provide an additional or alternative mechanism of tRNA binding dependent toxicity of mutant synthetases. This phenomenon could be observed with other aminoacyl-tRNA synthetases which possess a deacylation function.

The aspartic acid residue targeted for mutagenesis in the examples is conserved in all known isoleucyl- and methionyl-tRNA synthetases and in all but one (*N. crassa* leucyl-tRNA synthetase) of the known subgroup class I synthetases (Shiba, K. and P. Schimmel, Proc. Natl Acad. Sci. USA, 89: 1880-1884 (1992); Hou, Y.-M. et al., Proc.

Natl. Acad. Sci. USA, 88: 976-980 (1991)). It is predicted that mutagenesis of the corresponding aspartic acid in other synthetases, such as Asp85 (*Saccharomyces cerevisiae*), Asp87 (*Methanobacterium thermoautrophicum*), and Asp91 (*Tetrahymena thermophila*) of IleRS, and of Asp40 (*Thermus thermophilus*), Asp60 (*Saccharomyces cerevisiae* mitochondrial), Asp244 (*Saccharomyces cerevisiae* cytoplasmic), and Asp51 (*Bacillus stearothermophilus*) of MetRS (Ghosh, G. et al., Biochemistry, 30:9569-9575 (1991); Shiba, K. and P. Schimmel, Proc. Natl. Acad. Sci. USA, 89:1880-1884 (1992)) would lead to similarly inactive isoleucyl- and methionyl-tRNA synthetases. Analogous residues (i.e., at a position corresponding to position 96 of the wild-type *E. coli* isoleucyl-tRNA synthetase) can be identified in most of the other members (Leu, Val and Cys) of the class I subgroup by sequence alignment. In this regard, it is also noted that Lys732 is conserved as a basic residue in all published isoleucyl-tRNA synthetase sequences (Figure 1). Thus, double mutants analogous to the D96A/K732T IleRS mutant can be constructed in these IleRS genes. Derivatives of a selected tRNA binding mutant, such as the D96A/K732T IleRS double mutant, which are unable to bind tRNA are useful in demonstrating the tRNA-binding dependent nature of toxicity of the selected mutant. Furthermore, recent reports of single point mutations or deletions which selectively inactivate either tRNA binding or amino acid activation (Clarke, N.D. et al., Science, 240:521-523 (1988); Ghosh, G. et al., Biochemistry, 30:9569-9575 (1991); Ghosh, G. et al., Biochemistry, 29(9):2220-2225 (1990); Kim, S. and P. Schimmel, J. Biol. Chem., 267:15563-15567 (1992); Shepard, A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992)) suggest that additional single and double mutants be constructed and screened for tRNA-binding dependent

toxicity and specificity of action against microbial pathogens.

Utility

The specificity of inhibition of aminoacylation of microbial tRNA as compared with mammalian tRNA, indicates that specific tRNA-binding drugs can have the requisite specificity for therapeutic applications (pharmaceutical applications, including veterinary applications) in the treatment of microbial pathogens. Thus, tRNA binding molecules of the present invention can be used as antimicrobial agents in antimicrobial therapy or gene therapy against microbial infection of a selected host. tRNA binding-dependent reduction in growth rate or arrest of growth or cell division of microbial pathogens (cellular or viral) can ultimately lead to inviability, or can aid host defenses in effectively controlling and clearing infection. The induction of microbial cell death is particularly desirable when host defenses are impaired.

A variety of hosts are susceptible to microbial infection, including eukaryotic hosts such as plants (e.g., tobacco, rice, beans, corn, potatoes) and animals (e.g., mammals such as humans, cows, horses, goats, sheep, dogs, cats). Suitable microbial targets include bacteria, mycobacteria, *Chlamydia* species, *Pneumocystis* species, spirochetes, actinomycetes and fungi. For example, gram-positive and gram-negative bacteria, such as *Streptococcus*, *Staphylococcus*, *Neisseria*, *Listeria*, *Clostridium*, *Enterobacter*, *Proteus*, *Pseudomonas*, *Klebsiella*, *Salmonella*, *Shigella*, *Serratia*, and *Bacteroides* species, *Escherichia coli*, *Mycobacterium tuberculosis*, and *Mycobacterium leprae* can be suitable targets. Fungal pathogens such as *Candida*, *Coccidioides*, *Histoplasma*, *Aspergillus*, and *Cryptococcus* species can also be suitable targets. Plant pathogens include

bacterial pathogens such as *Pseudomonas* species and fungal pathogens, such as the blight fungus *Phytophthora infestans*. Viral infections are often considered to be microbial. In cases in which a virus encodes a specific tRNA or tRNA binding molecule, therapy according to the present invention may be possible. Effective therapy of a selected microbe depends upon obtaining the requisite therapeutic benefit (e.g., inhibition of microbial growth) without damaging toxicity to the host (i.e., specificity of action against the microbe). Thus, a given microbial infection can be treated according to the present invention when a tRNA binding molecule has the requisite toxicity against the microbial pathogen, while sparing the host.

One application of the invention is in controlling or killing microbial contaminants present in mammalian cell culture. In this embodiment, specific inhibition of growth of the microbial pathogen is attained with minimal toxicity to the cultured cells.

It will be appreciated that the therapeutic approach of the present invention has other applications. For example, the approach can be applied in the treatment of parasitic diseases (e.g., protozoan infections, such as amebiasis, giardiasis, leishmaniasis, malaria, trypanosomiasis or metazoan nematode, cestode or trematode infections, such as schistosomiasis), where a tRNA binding molecule with appropriately selective activity is available.

In another application of the approach, a tRNA binding molecule is introduced into target cells which have been introduced into the host for a selected purpose. For example, cells may be introduced into a host for immunization (i.e., to stimulate an immune response) or cells may be engineered for purposes of gene therapy and introduced into a host cell. Subsequently, it may

desirable to eradicate these cells. A tRNA binding molecule, capable of selective toxicity to the introduced target cells in the host, is introduced into the target cells. Although the introduced cells may be cells of the same species as the host (e.g., lymphocytes engineered for purposes of gene therapy, tumor cells, fetal cells, allografts), growth of these cells may be inhibited according to the present method, given a tRNA binding molecule with appropriately selective activity. In one variation of this embodiment, the cell introduced into the host is engineered to contain a tRNA whose function is essential to viability of the cell (e.g., because it is required for translation in general or because it is required for translation of a particular protein, such as one containing a nonsense mutation, which is essential to viability of the cell), yielding a target cell against which a selected tRNA binding molecule may be selectively toxic.

According to the present method, a tRNA binding molecule, such as an inactive mutant aminoacyl-tRNA synthetase or tRNA binding synthetase fragment is introduced into the microbial cell. In one embodiment, a nucleic acid encoding the mutant protein can be introduced into the microbial cell. Expression of the nucleic acid and production of the toxic tRNA binding molecule will inhibit growth of the microbial cell. For example, the nucleic acid can be introduced by means of an expression vector capable of directing the expression of the nucleic acid encoding the mutant protein. Suitable expression vectors, which may be inducible or constitutive, are available for a variety of microbial systems, including bacterial, mycobacterial, and fungal vectors. The vector may be delivered to the microbe by packaging in a phage or virus capable of delivering the vector to the microbial cell.

Alternatively, synthetically or recombinantly produced tRNA binding molecules can be administered to the host by a suitable route, either alone or in combination with another drug. A variety of routes of administration are possible including, but not necessarily limited to oral, dietary, topical, parenteral (e.g., intravenous, intramuscular, subcutaneous injection) routes of administration. Formulation of a tRNA binding molecule, such as a mutant aminoacyl-tRNA synthetase or a tRNA binding fragment of a synthetase, will vary according to the route of administration selected (e.g., solution, emulsion, capsule). An appropriate composition can be prepared in a physiologically acceptable vehicle or carrier. For solutions or emulsions, suitable carriers include, for example, aqueous or alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles can include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles can include various additives, preservatives, or fluid, nutrient or electrolyte replenishers (See, generally, Remington's Pharmaceutical Science, 16th Edition, Mack, Ed. 1980).

To facilitate uptake by microbial cells, the molecule can be fused to a moiety (e.g., a moiety capable of binding a specific surface molecule or receptor) which facilitates uptake by microbial cells. For parenteral administration, vesicles (e.g., microparticulates or colloidal carriers composed of proteins or lipids, such as liposomes) can facilitate delivery of drug to the microbial pathogen (see Langer, R., Science, 249: 1527-1533 (1990). Vesicles may be targeted passively, in which case, they a taken up naturally by cells that scavenge foreign microparticulates, such as reticuloendothelial cells (macrophages). Active targeting of vesicles is

accomplished by placing a recognition sequence (e.g., an antibody or other molecule capable of binding the target microbial cell) onto the vesicle to obtain efficient uptake by the microbial cell. Controlled release systems (e.g., polymeric systems) can also be used with protein-based tRNA binding therapeutics.

Certain pathogenic bacteria survive and multiply within the phagocytic cells of the host, including *Mycobacterium tuberculosis*, *Brucella abortus*, *Salmonella typhosa* and others. Passive targeting of a toxic tRNA binding molecule to the reticuloendothelial system (the total body pool of macrophages) by vesicles as described above can be used to treat these intracellular pathogens. In addition, a gene therapy approach may be indicated in treatment of a pathogen such as *Mycobacterium tuberculosis*. For example, a tRNA binding molecule of the present invention can be introduced into host phagocytic cells by means of a suitable vector (e.g., a retroviral vector). The vector will direct the constitutive or inducible expression of the tRNA binding molecule, which is then taken up by the intracellular pathogen. Various strategies to facilitate uptake by the pathogen are possible, including fusion of the tRNA binding molecule to a moiety which mediates uptake of the molecule by the pathogen. In the case of a plant pathogen, introduction of the tRNA binding molecule by engineering the host cells would be an appropriate strategy. A variety of plant vectors are available for transformation of plants.

Where the target is a viral pathogen, the tRNA binding molecule is also introduced into host cells by administering the tRNA binding molecule to the host or by introducing it into host cells via gene therapy. Once inside host cells, the tRNA binding molecule would specifically sequester and/or deacylate viral tRNA.

A therapeutically effective amount of a tRNA binding molecule of the present invention is administered or introduced into cells. A therapeutically effective amount is one which results in a level of tRNA binding molecule in the microbial pathogen sufficient to achieve the desired effect of inhibiting microbial growth, without undue toxicity to the host (e.g., which results in a level of tRNA binding molecule in the microbial pathogen sufficient for the molecule to be selectively toxic to the microbial pathogen present in the host). In the case of a virus, a therapeutically effective amount is one which results in a level of tRNA binding molecule in the host cell sufficient to achieve the desired effect of inhibiting viral growth, without undue toxicity to the host.

The present invention will now be illustrated by the following Examples, which are not intended to be limiting in any way.

## EXAMPLE 1

## tRNA-Binding Dependent Inhibition of Growth

### Bacterial strains and plasmids

*E. coli* K-12 strain TG1 (*supE, hsd*Δ5, *thi,* Δ(*lac-proAB*)/F′[*traD*36,*proAB*$^+$, *lacI*$^q$, *lacZ*ΔM15], from Amersham, UK) was used as a host strain for site-directed mutagenesis. *E. coli* K-12 strain MV1184 (*ara,* Δ[*lac-proAB*], *rspL, thi,* [ϕ80 *lacZ*ΔM15], Δ[*srl-recA*], 306::Tn10[*tet*$^r$]/*F′*[*traD*36, *proAB*$^+$, *lacI*$^q$, *lacZ*ΔM15]) was used as a host in tRNA synthetase expression experiments. These cells contain an F′ episome carrying the lacI$^q$ gene which gives low levels of lac promoter activity in the absence of IPTG. *E. coli* K-12 strain IQ844/pRMS711 (Δ*ileS*203::*kan*, *recA*56, *araD*139, Δ[*argF-lac*]*U*169, *rpsL*150,

*relA1*, *flbB5301*, *deoC1*, *ptsF25*, *rbsR*/F'[*lacI*$^q$ *lac*$^+$*pro*$^+$] (Shepard A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968(1992); Shiba, K. and P. Schimmel, J. Biol. Chem., 267:22703-22706 (1992)) was used as a tester strain for isoleucyl-tRNA synthetase activity. The strain contains a chromosomal deletion of the *ileS* gene and is propagated by the expression of wild type IleRS at 30°C from the temperature-sensitive maintenance plasmid pRMS711 (chloramphenicol') which cannot replicate at 42°C. *E. coli* K-12 strain MI1 (Iaccarino, M. and P. Berg, J. Bacteriology 105:527-537 (1971)), which carries a chromosomal mutation in the *ileS* gene was used as a host for the purification of inactive mutant IleRS. The *ileS* mutation present in this strain decreases amino acid binding of the chromosomally encoded protein to undetectable levels at low isoleucine concentrations (<100 μM), thus allowing for biochemical analysis of phagemid-encoded IleRS proteins expressed in this background.

The pKS21 phagemid (Shiba, K. and P. Schimmel, Proc. Natl. Acad. Sci. USA, 89:1880-1884 (1992)), a derivative of pTZ19R (Pharmacia), encodes wild-type IleRS and was used as a template for site-directed mutagenesis. The phagemid allows for inducible expression of IleRS from the lac promoter and contains the β-lactamase gene conferring ampicillin resistance.

Site-directed mutagenesis and complementation assays

Single stranded DNA was isolated from the pKS21 phagemid and used as a template for the introduction of the Asp96→Ala mutation via oligonucleotide-directed mutagenesis, using an Amersham oligonucleotide mutagenesis system (Amersham, UK). Resulting phagemids were sequenced and those containing only the desired mutation were selected. A double mutant was derived from the D96A

mutant plasmid by subcloning a *BsiWI-Eco47III* fragment containing a Lys732→Thr mutation (K732T) from pAS205 (Shepard, A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992)) into the pKS21 phagemid encoding the D96A mutant. The wild-type, D96A mutant, and D96A/K732T doubly mutant pKS21 phagemids were transformed into the tester strain IQ844/pRMS711 and tested for growth at the permissive (30°C) and non-permissive (42°C) temperatures.

In vivo expression experiments

Phagemids were transformed into MV1184 cells and overnight cultures were grown from single colonies and then diluted 1:150 into LB/ampicillin. Two separate cultures were grown at 37°C for each IleRS mutant. One of the cultures was induced with 1 mM IPTG (Sigma) at $O.D._{600}$ ≈ 0.250. An equal volume of water was added to the other. Growth was monitored for up to 11 hours by measuring cell densities by $OD_{600}$ on a Beckman DU-64 spectrophotometer.

Western blot analysis

Small scale samples were removed from each culture at the end of the time course. Cell densities were normalized and cells were lysed by boiling in gel loading buffer. Extracts were fractionated by SDS-PAGE on 7% gels. Purified IleRS was run as a reference. Proteins were transferred onto a Immobilon-P PVDF membrane using a Milliblot-Semi-Dry Blotting apparatus (Millipore). The blots were blocked in milk, incubated with rabbit anti-*E. coli* IleRS polyclonal antiserum (Starzyk, R.M. et al., Science, 237:1614-1618 (1987)) and then treated with donkey anti-rabbit IgG linked to horseradish peroxidase (Amersham, UK). The blots were developed using the ECL chemiluminescent system (Amersham, UK).

Enzyme purification and enzyme assays

One liter cultures of MV1184 cells harboring the pKS21 phagemid encoding wild-type IleRS were induced during log phase and grown to saturation. Protein purification was performed as described by Shepard et al. (Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992)). The inactive IleRS proteins encoded by D96A and D96A/K732T mutant pKS21 phagemids were purified in the same manner, but from one liter cultures of strain MI1. In these cultures, plasmid-encoded synthetase was overexpressed more than 10-fold in relation to chromosomally encoded enzymes (data not shown). Enzyme concentrations were determined by Bradford analysis (BioRad) using bovine serum albumin as a standard or by active site titration (Fersht, A.R. et al., Biochemistry, 14(1):1-4 (1975)).

Standard aminoacylation reactions were performed as described by Shepard et al. (Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992)), except that assays were performed at ambient temperature using 1 nM enzyme and 5 $\mu$M purified $tRNA^{Ile}$ (Subriden RNA). The aminoacylation reaction employing various amounts of mutant inhibitory IleRS was performed similarly using 2 nM wild-type IleRS, 100 nM purified $tRNA^{Ile}$ and 0 to 1 $\mu$M mutant IleRS. The reaction cocktail (everything except wild-type and mutant IleRS) was pre-incubated at ambient temperature. Mutant proteins were added and the sample was mixed. Wild-type protein was added soon after followed by further mixing. It was noticed that, if mutant proteins were allowed to pre-incubate in the reaction mixture for longer times (5-10 minutes), then their inhibitory effects increased. For results reported here we used short (<20 seconds) pre-incubation times. In order to detect small quantities of charged tRNA the specific activity of [$^3$H]-Ile was increased to 1670 cpm/pmole in these experiments.

Measurements of tRNA binding

The filter binding assay of Yarus and Berg (Yarus, M. and P. Berg, J. Mol. Biol., 42:171-189 (1969); Yarus, M. and P. Berg, Anal. Biochem., 35:450-465 (1970)) was used to measure the binding affinity of the wild-type and D96A mutant IleRS for charged [$^3$H]-Ile-tRNA$^{Ile}$. [$^3$H]-Ile-tRNA was prepared by charging tRNA with [$^3$H]-Ile as described above. The [$^3$H]-Ile-tRNA was isolated through a series of phenol/chloroform extractions and ethanol precipitations. The filter binding reaction mixture (100 $\mu$L) contained 20 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.15 M $NH_4Cl$, 100 $\mu$g/ml bovine serum albumin, 4 mM $MgCl_2$, and 25 nM IleRS. After pre-incubation at ambient temperature [$^3$H]-Ile-tRNA$^{Ile}$ was added at 25 to 5000 nM. The entire reaction was filtered through a 24 mm nitrocellulose disk (Schleicher and Schuell) prewashed in ice-cold 20 mM Tris-Cl (pH 7.5) and 150 mM KCl. After three 1 mL washes of the same solution, the filters were dried and counted in Betafluor (National Diagnostics). Data were subjected to Scatchard analysis (Creighton, T.E., 1984, In: Proteins, (W.H.Freeman and Co.: New York) pp., 343-344).

RESULTS

The D96A IleRS mutant is inactive

The activity of the D96A mutant was tested in a complementation assay in the *ileS* null tester strain IQ844/pRMS711. This tester strain carries a chromosomal null mutation in the ileS gene, and contains a copy of ileS on a temperature-sensitive maintenance plasmid. Mutant or wild-type phagemid was introduced into the tester strain by electroporation. Transformants were plated directly at the permissive (30°C) or nonpermissive (42°C) temperature and scored for growth. Although mutant phagemid readily produced transformants on plates at the

permissive temperature, no transformants were produced at 42°C. In contrast, wild-type phagemid produced equal numbers of transformants at nonpermissive and permissive temperatures. Loss of the pRMS711 maintenance plasmid at the non-permissive temperature was confirmed by assaying for chloramphenicol sensitivity (loss of the drug resistance marker carried by pRMS711) in complementing phagemid transformants. Thus, D96A mutation resulted in stably produced protein devoid of activity, as measured by its inability to complement the *ileS* null tester strain IQ844/pRMS711.

Dominant lethality by expression of a mutant isoleucyl-tRNA synthetase

To determine the effect of expression of the mutant tRNA synthetase on cell growth, mutant or wild-type IleRS genes were introduced by transformation into strain MV1184, which contains a wild-type chromosomal *ileS* allele. The growth of MV1184 cultures containing plasmids encoding the wild-type or D96A mutant were monitored under induced or uninduced conditions. Induction of wild-type IleRS had little or no effect on the growth of MV1184 cells, demonstrating that over-production of the enzyme per se is not toxic (Figure 2A). In contrast, induction of D96A IleRS stopped cell growth within two hours (Figure 2B).

Suppression of dominant lethality of Asp96Ala IleRS by a second distal mutation

The dominant lethality of the D96A mutant is most likely related to the ability of its intact C-terminus to bind $tRNA^{Ile}$. If the mutant tRNA synthetase, unable to catalyze aminoacylation, still bound its cognate $tRNA^{Ile}$ with near wild-type affinity, the mutant could sequester

tRNA from the endogenous wild-type synthetase and the cellular protein synthesis machinery. Further experiments were conducted to determine whether $tRNA^{Ile}$ is the in vivo target responsible for the toxicity of D96A IleRS.

A lysine to threonine mutation at position 732 (K732T) was placed in the C-terminal domain of the D96A mutant enzyme in order to disrupt the ability of the mutant to bind $tRNA^{Ile}$. The K732T mutation increases the $K_m$ for $tRNA^{Ile}$ in the aminoacylation reaction by 225-fold (Shepard, A. et al., Proc. Natl. Acad. Sci. USA, 89:9964-9968 (1992)). The resulting double mutant D96A/K732T IleRS also accumulated in vivo but was unable to complement the IQ844/pRMS711 null strain.

Introduction of the C-terminal K732T mutation into the D96A mutant IleRS relieved almost all of the inhibitory effects of mutant IleRS expression on the growth of MV1184 cells (Figure 2C). Western blot analysis showed that the D96A and D96A/K732T mutant proteins were expressed at approximately equal levels. Thus, disruption of the capacity of the D96A mutant enzyme to bind $tRNA^{Ile}$ relieved its dominant in vivo toxicity.

Mutant IleRS proteins were expressed from multiple copy plasmids (15-20 copies/cell) under the control of the inducible *lac* promoter. Under these conditions, a concentration of mutant protein relative to the chromosomally encoded wild-type protein sufficient to be toxic to the microbial cell was obtained. Toxicity due to sequestration of $tRNA^{Ile}$ probably requires overexpression of the mutant synthetase, because in vivo ratios of $tRNA^{Ile}$:IleRS for *E. coli* are about 4 to 1 (Jakubowski, H. and E. Goldman, J. Bacteriol., 158(3):769-776 (1984)). Therefore, a mutant IleRS, with similar tRNA binding capacity, would need to be overexpressed at least 4-fold relative to endogenous *E. coli* synthetase in order to

titrate a significant amount of $tRNA^{Ile}$ in vivo sufficient to achieve the desired effect of inhibiting microbial growth.

In vitro characterization of mutant proteins

The pKS21-encoded D96A and D96A/K732T mutant enzymes were expressed and purified from MI1 cells, which contain a mutant chromosomal *ileS* allele (see above). The MI1-encoded IleRS has a high $K_m$ for isoleucine (>100 μM) and is able to maintain cell viability in vivo only when cell media are supplemented with isoleucine (Iaccarino, M. and P. Berg, J. Bacteriol., 105:527-537 (1971)). The activity of the MI1 mutant enzyme is not detectable under normal in vitro assay conditions which employ 20 μM isoleucine. This feature of the strain permits the direct biochemical analysis of mutant proteins upon over-production and purification from MI1 cells.

Figure 3 shows that, under the conditions of the aminoacylation assay, no activity could be detected for either the D96A mutant or the MI1 endogenous mutant enzyme. Additional experiments showed that the D96A mutant had a severely reduced $k_{cat}$ for aminoacyl-adenylate formation.

A nitrocellulose filter binding assay was employed to determine the apparent dissociation constant of the IleRS·Ile-$tRNA^{Ile}$ complex at pH 7.5. The aminoacylated form of $tRNA^{Ile}$ was used for these experiments because it is the predominant form of tRNA in vivo (Yegian, C.D. et al., Proc. Natl. Acad. Sci. USA, 55:839-846 (1966)) and the amino acid provided a convenient radiolabel. These experiments indicated similar dissociation constants of 0.33 μM and 0.48 μM for the wild-type and D96A mutant binding complexes, respectively. The double mutant

7.5), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 M KCl, 1 mM EDTA, 0.25 M sucrose, and 15% glycerol. The suspension was centrifuged at 30,000 rpm for 45 minutes. The resulting supernatant was diluted 5-fold with phosphate buffer (10 mM potassium phosphate (pH 7.5), 20 mM β-mercaptoethanol, 1 mM EDTA, 4 mM $MgCl_2$, 15% glycerol, and 0.1% PMSF) and loaded onto a DEAE-cellulose column equilibrated in phosphate buffer. The column was washed with 60 ml of the same buffer. Proteins were eluted from the column with 0.25 KCl, 10mM potassium phosphate (pH 6.5), 1 mM β-mercaptoethanol, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ATP, 50% glycerol, and 0.1% PMSF. Fractions were collected and $OD_{280}$ was measured for each fraction. Fractions containing a high concentration of protein were used for charging assays. Fractionated extracts were stored at -20°C.

Aminoacylation Assays

Aminoacylation assays were performed essentially as described in Example 1, except that a HeLa cell extract and crude calf liver (bovine) tRNA were used. To determine the effect of mutant *E. coli* IleRS proteins on aminoacylation, the assay was performed in the presence of 1.0 μM D96A IleRS protein or 1.0 μM D96A/K732T IleRS protein.

RESULTS

In contrast to the results of the aminoacylation assays using *E. coli* IleRS and *E. coli* $tRNA^{Ile}$ presented in Example 1, aminoacylation of crude tRNA with isoleucine by a HeLa cell extract in the presence of 1.0 μM D96A IleRS was largely unaffected (Figure 5). Consistent with the conclusion that the D96A mutant has little if any effect on aminoacylation by the mammalian enzyme, the extent of

D96A/K732T IleRS had a $K_d$ for complex dissociation which was too high (> 3 $\mu$M) to be measured by this assay.

Inhibition of in vitro aminoacylation by Asp96Ala but not Asp96Ala/Lys732Thr mutant IleRS

The mutant IleRS proteins, purified from MI1 cells, were used in in vitro charging assays designed to mimic the in vivo expression studies. The aminoacylation activity of catalytic amounts of wild-type enzyme was measured in the presence of substrate levels of D96A or D96A/K732T protein. A prediction of the tRNA sequestering hypothesis is that, as the concentration of the D96A mutant protein is increased in concentration through its $K_d$ for tRNA, the available free $tRNA^{Ile}$ is decreased, leading to inhibition of charging. The D96A mutant, but not the D96A/K732T double mutant, significantly inhibited aminoacylation over the concentration range investigated (Figure 4A and 4B).

## EXAMPLE 2

## Selectivity of Inhibition of Aminoacylation

In order to determine whether the mutant IleRS proteins were selective in inhibition of aminoacylation, the effect of the D96A and D96A/K732T IleRS proteins on aminoacylation by a mammalian isoleucyl-tRNA synthetase was determined.

Preparation of HeLa Cell Extracts

HeLa cell extracts were prepared after the method of Pearson et al. (Pearson et al., Biochim. Biophys. Acta, 294:236 (1973); describing preparation of crude extracts from calf liver). The HeLa cell pellet from a one liter culture was resuspended in 10 ml of 0.5 M Tris-Cl (pH

aminoacylation in the presence of the D96A IleRS mutant was observed to be similar to the extent of aminoacylation in the presence of the non-binding D96A/K732T IleRS mutant. Other experiments verified that *E. coli* IleRS did not aminoacylate calf liver tRNA under the conditions of the assay. Additional aminoacylation assays were performed using crude *E. coli* tRNA and *E. coli* IleRS under identical conditions. In these assays, the D96A IleRS mutant inhibited aminoacylation by 65% and the D96A/K732T IleRS mutant inhibited aminoacylation by 15%, indicating that specific inhibition can be observed using crude or fractionated tRNA in the *E. coli* system. These results indicate that species specific inhibition of microbial tRNA function can be achieved with tRNA binding molecules.

## EXAMPLE 3

## Identification of an Amino Acid Binding Site in a Class I Aminoacyl-tRNA Synthetase

*E. coli* isoleucyl-tRNA synthetase (IleRS) is a 939 amino acid enzyme which catalyzes the specific attachment of isoleucine to $tRNA^{Ile}$. The enzyme must discriminate between the amino acids isoleucine and valine based solely on the extra methylene group of isoleucine. In order to identify residues of IleRS responsible for amino acid binding and the strong preference of the enzyme for isoleucine over valine, affinity labeling of active site peptides and mutagenesis of possible critical residues in these peptides was carried out. In addition, a mutation of IleRS which confers isoleucine auxotrophy in an *E. coli* strain has been identified.

Affinity labeling and site-directed mutagenesis

The affinity label bromoacetylated-Ile-$tRNA^{Ile}$ (Santi, D. et al., Biochem. Biophys. Res. Commun., 51: 370-375) was used to probe for residues interacting with the isoleucyl moiety of charged $tRNA^{Ile}$. Two tryptic peptides beginning with $Thr^{50}$ and $Ile^{452}$ were identified as being reactive toward the affinity label.

The peptide beginning with $Thr^{50}$ contains the highly conserved signature sequence ending in HIGH (His-Ile-Gly-His, according to the standard single-letter amino acid code) found in class I synthetases, as well as a conserved proline previously identified as important in methionine binding by the class I methionyl-tRNA synthetase (Burbaum, J. and P. Schimmel, Protein Science, 1:575-581 (1992)). Mutagenesis of other residues in the peptide has identified a Gly56→Ala mutation (G56A) which increases the $K_m$ for amino acid binding by 1750-fold (Table 2). Further mutagenesis of residues in the tryptic peptide beginning with $Thr^{50}$ may identify additional residues involved in isoleucine binding.

The peptide beginning with $Ile^{452}$ contains a conserved peptide sequence DWCISR (Asp-Trp-Cys-Ile-Ser-Arg, according to the standard single-letter amino acid code), which contains a cysteine residue previously affinity labeled by an isoleucine analog (Rainey, et al., Eur. J. Biochem., 63: 419-426). Extensive mutagenesis of the region of the IleRS gene encoding this peptide has led to the identification of several residues critical for amino acid binding and transfer of the activated amino acid to $tRNA^{Ile}$. Several conservative point mutations in the DWCISR sequence significantly reduced enzymatic activity. Residues especially sensitive to change include $Trp^{462}$, $Arg^{466}$, and $Arg^{468}$. The results of the biochemical characterization of Trp462→Phe (W462F), Arg466→Gln

(R466Q), and Arg468→Gln (R468Q) IleRS mutant proteins are shown in Table 2 below.

These results establish that the tryptic peptides of IleRS identified by these cross-linking studies correspond to regions of IleRS that can be targeted by mutagenesis to construct IleRS proteins defective in amino acid binding. The mutant IleRS proteins shown in Table 2 and other similar mutants can be over-expressed in a microbial cell (e.g., *E. coli*) from an appropriate vector, such as an inducible expression vector, and toxicity can be determined. The Trp462→Phe (W462F), Arg466→Gln (R466Q), and Arg468→Gln (R468Q) IleRS mutant proteins retain the deacylation or editing function which can contribute to toxicity, suggesting that they bind tRNA, and are candidate toxic tRNA binding molecules with antimicrobial utility.

TABLE 2

BIOCHEMICAL ANALYSIS OF MUTATIONS IN CROSS-LINKED PEPTIDES

| Mutant | Complementation[A] | Km (Ile) | Km (Val) | Km (ATP) | Editing | Aminoacylation[B] |
|---|---|---|---|---|---|---|
| Wild-type | ++++ | 4 μM | 450 μM | 460 μM | +++ | +++ |
| Mutations made in cross-linked peptide 1 | | | | | | |
| G56A | +/- | 7 mM | 7 mM | 3600 μM | +++ | Undetermined |
| Mutations made in or near cross-linked peptide 2 | | | | | | |
| W462F | +/- | 290 μM | | 3400 μM | +++ | +[C] |
| R466Q | - | 200 μM | | 5300 μM | +++ | +[C] |
| R468Q | + | 12 μM | 1400 μM | 730 μM | +++ | +[C] |

[A] Determined as in Example 1.

[B] These conditions were under high isoleucine concentrations in order to isolate the transfer reaction.

[C] At least 100-fold less than wild-type.

Editing activity of Gly56→Ala mutant

As noted above the Gly56→Ala mutation (G56A) led to a 1750-fold increase in the $K_m$ for amino acid binding. Interestingly, an increase in the value for $K_m$ for valine from 450 μM in the wild type IleRS to 7 mM in the G56A mutant was also observed (Table 2). This observation indicates that the Gly56→Ala substitution eliminates discrimination between isoleucine and valine in the amino acid activation reaction. However, the hydrolytic editing functions of the mutant enzyme are intact.

When presented with $tRNA^{Ile}$, enzyme-bound misactivated valyl-adenosine monophosphate (Val-AMP) is hydrolyzed either (i) directly or (ii) by transient formation of Val-$tRNA^{Ile}$ followed by rapid deacylation of the mischarged species (Norris, A.T. and P. Berg, Proc. Natl. Acad. Sci. USA, 52: 330 (1964); Baldwin, A.N. and P. Berg, J. Biol. Chem., 241: 839 (1966); Schreier, A.A. and P.R. Schimmel, Biochemistry, 11: 1582 (1972); Eldred, E.W. and P.R. Schimmel, J. Biol. Chem., 247: 2961 (1972); Fersht, A.R., Biochemistry, 16: 1025 (1977)). These pre- and post-transfer editing reactions are manifested by a $tRNA^{Ile}$-dependent hydrolysis of ATP in the presence of valine. The overall pre- and post-transfer ATP hydrolysis induced by the addition of $tRNA^{Ile}$ was measured for the wild-type and G56A mutant enzymes in the presence of valine.

Mutant or wild-type enzyme was isolated as described in Example 1. Reactions contained 150 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 200 mM valine, 3 mM [$\gamma$-$^{32}$P]ATP (10-20 cpm/pmol), pyrophosphatase (2 U/ml), 14 μM $tRNA^{Ile}$, and 2.8 μM enzyme. Reactions were assayed at 25 °C for up to 20 minutes and were quenched with four volumes of 7% $HClO_4$. Activated charcoal containing 10 mM sodium pyrophosphate was added, and ATP was separated by filtration through glass fiber pads (Schleicher and Schuell).

ATP consumption (pmol ATP remaining/pmol enzyme) in the presence or absence of $tRNA^{Ile}$ was determined over time (minutes). It was determined that the mutant enzyme was as active as the wild-type enzyme in overall pre- and post-transfer ATP hydrolysis induced by the addition of $tRNA^{Ile}$. In particular, the $tRNA^{Ile}$-dependent rate of hydrolysis of Val-AMP was measured as 2.7 $s^{-1}$ in both cases. (Although the G56A mutant showed more ATP hydrolysis in the absence of $tRNA^{Ile}$ than did the wild-type enzyme when the assay was carried out for longer time periods, this hydrolysis represented only 15% or less of the $tRNA^{Ile}$-dependent hydrolysis by the mutant.)

In addition, the IleRS-catalyzed hydrolysis of mischarged Val-$tRNA^{Ile}$ was assessed (Eldred, E.W. and P.R. Schimmel, J. Biol. Chem., 247: 2961 (1972)). *Bacillus stearothermophilus* valyl-tRNA synthetase was purified from *E. coli* strain MV1184 harboring plasmid pTB8, which encodes the *B. stearothermophilus* ValRS (Borgford, T.J. *et al.*, Biochemistry, 26: 2480 (1987)). *Bacillus stearothermophilus* ValRS was used to charge *E. coli* $tRNA^{Ile}$ with [$^3$H]valine (Giegé, R. *et al.*, Eur. J. Biochem., 45: 351 (1974)). The misacylated [$^3$H]valine-$tRNA^{Ile}$ was purified through a series of phenol-chloroform extractions and ethanol precipitations. Deacylation reactions, performed at 25 °C, contained 150 mM tris-HCl (pH 7.5), 10 mM $MgCl_2$, 3.25 $\mu$M [$^3$H]valine-$tRNA^{Ile}$, (2170 cpm/pmol), pyrophosphatase (4 U/ml), and 5.2 nM enzyme. Aliquots of the reaction mixture were quenched on Whatman 3MM filter pads soaked in 5% trichloroacetic acid (TCA) and then washed repeatedly in 5% TCA followed by 100% ethanol to remove free [$^3$H]valine. Under these conditions, the spontaneous rate of valine-$tRNA^{Ile}$ hydrolysis was less than 0.0002 $s^{-1}$.

The post-transfer IleRS-catalyzed deacylation of Val-tRNA$^{Ile}$ was not diminished in the mutant. If anything, this activity appeared slightly higher for the mutant enzyme as compared with the wild-type enzyme (wild-type $k_{cat}$ = 0.07 $s^{-1}$; G56A mutant $k_{cat}$ = 0.13 $s^{-1}$).

Identification of a mutation which confers an isoleucine auxotrophy upon *E. coli*

*E. coli* strain MI1 (Iaccarino, M. and P. Berg, J. Bacteriol., 105: 527 (1971); Treiber, G. and M. Iaccarino, J. Bacteriol., 107: 828-832 (1971)) contains a chromosomal mutation in the *IleS* gene which is responsible for the isoleucine requirement of the strain. The *IleS* mutant gene was amplified using PCR, and a single amino acid substitution (Phe570→Ser or F570S) has been identified as the likely cause of auxotrophy. Phe$^{570}$ is within the nucleotide-binding fold at the beginning of the fourth (or D) helix, and, on the basis of the alignment of this region with methionine tRNA synthetase (Shiba, K. and P. Schimmel, Proc. Natl. Acad. Sci. U.S.A., 89: 1880 (1992)), also contributes to the formation of the amino acid binding site (Ghosh, G. *et al.*, Biochemistry, 30: 9569 1991)). When the F570S mutation is engineered into an otherwise wild-type IleRS, the resulting mutant enzyme has a $K_m$ for isoleucine which is elevated ~2000-fold as compared with the wild-type enzyme (Table 3). The $K_m$ for valine was greater than 200 mM. In addition, the ratio of the $k_{cat}/K_m$ for isoleucine to the $k_{cat}/K_m$ for valine for the mutant enzyme ($k_{cat}/K_m$ (Ile) ÷ $k_{cat}/K_m$ (Val) = 230) was at least as great as the corresponding ratio observed for the wild-type enzyme ($k_{cat}/K_m$ (Ile) ÷ $k_{cat}/K_m$ (Val) = 180), indicating that this mutant retains the ability to discriminate against valine. Post-transfer editing activity was also largely unimpaired for the F570S mutant,

which was observed to have a $k_{cat}$ for deacylation of Val-tRNA$^{Ile}$ of 0.17 $s^{-1}$ as compared with 0.07 $s^{-1}$ for wild-type IleRS. Together, the F570S and G56A mutants illustrate that mutations which have different effects on the amino acid binding site of the enzyme can have little or no effect on the editing reactions catalyzed by IleRS.

Residues corresponding to the phenylalanine at position 570 (Phe$^{570}$) of the *E. coli* IleRS can be identified by sequence similarity to other IleRS enzymes; phenylalanine is strictly conserved among all known isoleucyl-tRNA synthetases and is not found in any other class I tRNA synthetase. It is likely that Phe$^{570}$ forms part of the amino acid binding pocket of IleRS that specifically accommodates the isoleucine side chain. Construction of similar substitutions (e.g., Phe to Ser) at the corresponding residue in other IleRS proteins is predicted to cause analogous amino acid binding defects. Construction of mutations close to or adjacent to position 570 in *E. coli* IleRS, or to the corresponding residue in other IleRS proteins, may yield other mutants defective in tRNA binding.

TABLE 3

BIOCHEMICAL ANALYSIS OF F570S MUTANT IleRS

| Mutant | Complementation | Km (Ile) | Km (Val) | Km (ATP) | Editing | Aminoacylation |
|---|---|---|---|---|---|---|
| Wild-type | ++++ | 4 μM | 450 μM | 460 μM | +++ | +++ |
| F570S | ++ | 8 mM | >200 mM | 1020 μM | +++ | (+-+++)* |

* Not determinable in vitro; aminoacylation inferred from complementation activity.

Dominant lethality by expression of mutant isoleucyl-tRNA synthetases

In order to determine the effect of expression of the mutant tRNA synthetases on cell growth, genes encoding the Gly56→Ala (G56A) mutant or the Phe570→Ser (F570S) mutant were introduced by transformation into strain MV1184, which contains a wild-type chromosomal *ileS* allele. The growth of MV1184 cultures containing plasmids encoding the G56A or F570S mutants were monitored essentially as described in Example 1, under both induced (+ 500 μM IPTG induction at 3.0 hours) or uninduced conditions. Induction of expression of either the G56A or the F570S IleRS mutant stopped cell growth, although the effect of the G56A mutant was more pronounced (Figure 6). Toxicity to microbial cells by over-expression of the F570S mutant may be enhanced under conditions where, for example, isoleucine is limiting.

Equivalents

Those skilled in the art will be able to recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

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(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
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(A) APPLICATION NUMBER: US 08/068,382
(B) FILING DATE: 28-MAY-1993

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ser Gly Tyr Asp Ser Pro Tyr Val Pro Gly Trp Asp Cys His Gly Leu
1 5 10 15

Pro

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Thr Gly His His Val Glu Arg Arg Phe Gly Trp Asp Thr His Gly Val
1 5 10 15

Pro

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Arg Gly Phe Asn Val Arg Arg Gln Pro Gly Trp Asp Thr His Gly Leu
1 5 10 15

Pro

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asn Gly Lys Tyr Val Glu Arg Arg Phe Gly Trp Asp Cys His Gly Leu
1 5 10 15

Pro

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Asp Gly Tyr Arg Thr Phe Phe Leu Thr Gly Thr Asp Glu His Gly Glu
1 5 10 15

Thr

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Gly Asn Leu Ser Phe Phe Thr Thr Gly Thr Asp Glu His Gly Leu
1 5 10 15

Lys

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Arg Asn Tyr Asn Ala Leu Phe Ile Cys Gly Thr Asp Glu Tyr Gly Thr
1 5 10 15

Ala

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg Gly Tyr Asp Val Met Tyr Leu Thr Gly Thr Asp Glu His Gly Gln
1 5 10 15

Lys

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Gly His Glu Val Asn Phe Ile Cys Ala Asp Asp Ala His Gly Thr
1 5 10 15

Pro

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ser Phe Tyr Leu Gln Ile Ile Lys Asp Arg Gln Tyr
1 5 10

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Asn Trp Tyr Ile Arg Phe Asn Arg Arg Arg Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Arg Trp Tyr Ile Arg Leu Ile Arg Ser Arg Thr Trp
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Asn Trp Tyr Ile Arg Leu Asn Arg Asn Arg Leu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Asn Arg Tyr Ile Asn Glu Lys Lys Pro Trp Glu Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

Asn Thr Leu Val Gln Asn Ser Lys Pro Trp Glu Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Asn Gln Phe Leu Gln Glu Asn Lys Leu Asp Asn Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Asn Lys Tyr Ile Asp Glu Thr Gln Pro Trp Val Leu
1 5 10

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Asn Arg Tyr Val Asp Glu Gln Ala Pro Trp Val Val
1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2820 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2820

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
ATG AGT GAC TAT AAA TCA ACC CTG AAT TTG CCG GAA ACA GGG TTC CCG        48
Met Ser Asp Tyr Lys Ser Thr Leu Asn Leu Pro Glu Thr Gly Phe Pro
 1               5                  10                  15

ATG CGT GGC GAT CTC GCC AAG CGC GAA CCC GGA ATG CTG GCG CGT TGG        96
Met Arg Gly Asp Leu Ala Lys Arg Glu Pro Gly Met Leu Ala Arg Trp
            20                  25                  30

ACT GAT GAT GAT CTG TAC GGC ATC ATC CGT GCG GCT AAA AAA GGC AAA       144
Thr Asp Asp Asp Leu Tyr Gly Ile Ile Arg Ala Ala Lys Lys Gly Lys
        35                  40                  45

AAA ACC TTC ATT CTG CAT GAT GGC CCT CCT TAT GCG AAT GGC AGC ATT       192
Lys Thr Phe Ile Leu His Asp Gly Pro Pro Tyr Ala Asn Gly Ser Ile
    50                  55                  60
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CAT ATT GGT CAC TCG GTT AAC AAG ATT CTG AAA GAC ATT ATC GTG AAG      240
His Ile Gly His Ser Val Asn Lys Ile Leu Lys Asp Ile Ile Val Lys
 65                  70                  75                  80

TCC AAA GGG CTT TCC GGT TAT GAC TCG CCG TAT GTG CCT GGC TGG GAC      288
Ser Lys Gly Leu Ser Gly Tyr Asp Ser Pro Tyr Val Pro Gly Trp Asp
                 85                  90                  95

TGC CAC GGT CTG CCG ATC GAG CTG AAA GTC GAG CAA GAA TAC GGT AAG      336
Cys His Gly Leu Pro Ile Glu Leu Lys Val Glu Gln Glu Tyr Gly Lys
            100                 105                 110

CCG GGT GAG AAA TTC ACC GCC GCC GAG TTC CGC GCC AAG TGC CGC GAA      384
Pro Gly Glu Lys Phe Thr Ala Ala Glu Phe Arg Ala Lys Cys Arg Glu
        115                 120                 125

TAC GCG GCG ACC CAG GTT GAC GGT CAA CGC AAA GAC TTT ATC CGT CTG      432
Tyr Ala Ala Thr Gln Val Asp Gly Gln Arg Lys Asp Phe Ile Arg Leu
    130                 135                 140

GGC GTG CTG GGC GAC TGG TCG CAC CCG TAC CTG ACC ATG GAC TTC AAA      480
Gly Val Leu Gly Asp Trp Ser His Pro Tyr Leu Thr Met Asp Phe Lys
145                 150                 155                 160

ACT GAA GCC AAC ATC ATC CGC GCG CTG GGC AAA ATC ATC GGC AAC GGT      528
Thr Glu Ala Asn Ile Ile Arg Ala Leu Gly Lys Ile Ile Gly Asn Gly
                165                 170                 175

CAC CTG CAC AAA GGC GCG AAG CCA GTT CAC TGG TGC GTT GAC TGC CGT      576
His Leu His Lys Gly Ala Lys Pro Val His Trp Cys Val Asp Cys Arg
            180                 185                 190

TCT GCG CTG GCG GAA GCG GAA GTT GAG TAT TAC GAC AAA ACT TCT CCG      624
Ser Ala Leu Ala Glu Ala Glu Val Glu Tyr Tyr Asp Lys Thr Ser Pro
        195                 200                 205

TCC ATC GAC GTT GCT TTC CAG GCA GTC GAT CAG GAT GCA CTG AAA GCA      672
Ser Ile Asp Val Ala Phe Gln Ala Val Asp Gln Asp Ala Leu Lys Ala
    210                 215                 220

AAA TTT GCC GTA AGC AAC GTT AAC GGC CCA ATC TCG CTG GTA ATC TGG      720
Lys Phe Ala Val Ser Asn Val Asn Gly Pro Ile Ser Leu Val Ile Trp
225                 230                 235                 240

ACC ACC ACG CCG TGG ACT CTG CCT GCC AAC CGC GCA ATC TCT ATT GCA      768
Thr Thr Thr Pro Trp Thr Leu Pro Ala Asn Arg Ala Ile Ser Ile Ala
                245                 250                 255

CCA GAT TTC GAC TAT GCG CTG GTG CAG ATC GAC GGT CAG GCC GTG ATT      816
Pro Asp Phe Asp Tyr Ala Leu Val Gln Ile Asp Gly Gln Ala Val Ile
            260                 265                 270

CTG GCG AAA GAT CTG GTT GAA AGC GTA ATG CAG CGT ATC GGC GTG ACC      864
Leu Ala Lys Asp Leu Val Glu Ser Val Met Gln Arg Ile Gly Val Thr
        275                 280                 285

GAT TAC ACC ATT CTC GGC ACG GTA AAA GGT GCG GAC GTT GAG CTG CTG      912
Asp Tyr Thr Ile Leu Gly Thr Val Lys Gly Ala Asp Val Glu Leu Leu
    290                 295                 300
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CGC TTT ACC CAT CCG TTT ATG GGC TTC GAC GTT CCG GCA ATC CTC GGC          960
Arg Phe Thr His Pro Phe Met Gly Phe Asp Val Pro Ala Ile Leu Gly
305                 310                 315                 320

GAT CAC GTT ACC CTG GAT GCC GGT ACC GGT GCC GTT CAC ACC GCG CCT         1008
Asp His Val Thr Leu Asp Ala Gly Thr Gly Ala Val His Thr Ala Pro
                325                 330                 335

GGC CAC GGC CCG GAC GAC TAT GTG ATC GGT CAG AAA TAC GGC CTG GAA         1056
Gly His Gly Pro Asp Asp Tyr Val Ile Gly Gln Lys Tyr Gly Leu Glu
            340                 345                 350

ACC GCT AAC CCG GTT GGC CCG GAC GGC ACT TAT CTG CCG GGC ACT TAT         1104
Thr Ala Asn Pro Val Gly Pro Asp Gly Thr Tyr Leu Pro Gly Thr Tyr
        355                 360                 365

CCG ACG CTG GAT GGC GTG AAC GTC TTC AAA GCG AAC GAC ATC GTC GTT         1152
Pro Thr Leu Asp Gly Val Asn Val Phe Lys Ala Asn Asp Ile Val Val
    370                 375                 380

GCG CTG CTG CAG GAA AAA GGC GCG CTG CTG CAC GTT GAG AAA ATG CAG         1200
Ala Leu Leu Gln Glu Lys Gly Ala Leu Leu His Val Glu Lys Met Gln
385                 390                 395                 400

CAC AGC TAT CCG TGC TGC TGG CGT CAC AAA ACG CCG ATC ATC TTC CGC         1248
His Ser Tyr Pro Cys Cys Trp Arg His Lys Thr Pro Ile Ile Phe Arg
                405                 410                 415

GCG ACG CCG CAG TGG TTC GTC AGC ATG GAT CAG AAA GGT CTG CGT GCG         1296
Ala Thr Pro Gln Trp Phe Val Ser Met Asp Gln Lys Gly Leu Arg Ala
            420                 425                 430

CAG TCA CTG AAA GAG ATC AAA GGC GTG CAG TGG ATC CCG GAC TGG GGC         1344
Gln Ser Leu Lys Glu Ile Lys Gly Val Gln Trp Ile Pro Asp Trp Gly
        435                 440                 445

CAG GCG CGT ATC GAG TCG ATG GTT GCT AAC CGT CCT GAC TGG TGT ATC         1392
Gln Ala Arg Ile Glu Ser Met Val Ala Asn Arg Pro Asp Trp Cys Ile
    450                 455                 460

TCC CGT CAG CGC ACC TGG GGT GTA CCG ATG TCA CTG TTC GTG CAC AAA         1440
Ser Arg Gln Arg Thr Trp Gly Val Pro Met Ser Leu Phe Val His Lys
465                 470                 475                 480

GAC ACG GAA GAG CTG CAT CCG CGT ACC CTT GAA CTG ATG GAA GAA GTG         1488
Asp Thr Glu Glu Leu His Pro Arg Thr Leu Glu Leu Met Glu Glu Val
                485                 490                 495

GCA AAA CGC GTT GAA GTC GAT GGC ATC CAG GCG TGG TGG GAT CTC GAT         1536
Ala Lys Arg Val Glu Val Asp Gly Ile Gln Ala Trp Trp Asp Leu Asp
            500                 505                 510

GCG AAA GAG ATC CTC GGC GAC GAA GCT GAT CAG TAC GTG AAA GTG CCG         1584
Ala Lys Glu Ile Leu Gly Asp Glu Ala Asp Gln Tyr Val Lys Val Pro
        515                 520                 525

GAC ACA TTG GAT GTA TGG TTT GAC TCC GGA TCT ACC CAC TCT TCT GTT         1632
Asp Thr Leu Asp Val Trp Phe Asp Ser Gly Ser Thr His Ser Ser Val
    530                 535                 540
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GTT GAC GTG CGT CCG GAA TTT GCC GGT CAC GCA GCG GAC ATG TAT CTG      1680
Val Asp Val Arg Pro Glu Phe Ala Gly His Ala Ala Asp Met Tyr Leu
545                 550                 555                 560

GAA GGT TCT GAC CAA CAC CGC GGC TGG TTC ATG TCT TCC CTA ATG ATC      1728
Glu Gly Ser Asp Gln His Arg Gly Trp Phe Met Ser Ser Leu Met Ile
                565                 570                 575

TCC ACC GCG ATG AAG GGT AAA GCG CCG TAT CGT CAG GTA CTG ACC CAC      1776
Ser Thr Ala Met Lys Gly Lys Ala Pro Tyr Arg Gln Val Leu Thr His
            580                 585                 590

GGC TTT ACC GTG GAT GGT CAG GGC CGC AAG ATG TCT AAA TCC ATC GGC      1824
Gly Phe Thr Val Asp Gly Gln Gly Arg Lys Met Ser Lys Ser Ile Gly
        595                 600                 605

AAT ACC GTT TCG CCG CAG GAT GTG ATG AAC AAA CTG GGC GCG GAT ATT      1872
Asn Thr Val Ser Pro Gln Asp Val Met Asn Lys Leu Gly Ala Asp Ile
    610                 615                 620

CTG CGT CTG TGG GTG GCA TCA ACC GAC TAC ACC GGT CAA ATG GCC GTT      1920
Leu Arg Leu Trp Val Ala Ser Thr Asp Tyr Thr Gly Gln Met Ala Val
625                 630                 635                 640

TCT GAC GAG ATC CTG AAA CGT GCT GCC GAT AGC TAT CGT CGT ATC CGT      1968
Ser Asp Glu Ile Leu Lys Arg Ala Ala Asp Ser Tyr Arg Arg Ile Arg
                645                 650                 655

AAC ACC GCG CGC TTC CTC CTG GCA AAC CTG AAC GGT TTT GAT CCA GCA      2016
Asn Thr Ala Arg Phe Leu Leu Ala Asn Leu Asn Gly Phe Asp Pro Ala
            660                 665                 670

AAA GAT ATG GTG AAA CCG GAA GAG ATG GTG GTA CTG GAT CGC TGG GCC      2064
Lys Asp Met Val Lys Pro Glu Glu Met Val Val Leu Asp Arg Trp Ala
        675                 680                 685

GTA GGT TGT GCG AAA GCG GCA CAG GAA GAC ATC CTC AAG GCG TAC GAA      2112
Val Gly Cys Ala Lys Ala Ala Gln Glu Asp Ile Leu Lys Ala Tyr Glu
    690                 695                 700

GCA TAC GAT TTC CAC GAA GTG GTA CAG CGT CTG ATG CGC TTC TGC TCC      2160
Ala Tyr Asp Phe His Glu Val Val Gln Arg Leu Met Arg Phe Cys Ser
705                 710                 715                 720

GTT GAG ATG GGT TCC TTC TAC CTC GAC ATC ATC AAA GAC CGT CAG TAC      2208
Val Glu Met Gly Ser Phe Tyr Leu Asp Ile Ile Lys Asp Arg Gln Tyr
                725                 730                 735

ACG CCA AAG CGG ACA GTG TGG GCG CGT CGT AGC TGC CAG ACT GCG CTA      2256
Thr Pro Lys Arg Thr Val Trp Ala Arg Arg Ser Cys Gln Thr Ala Leu
            740                 745                 750

TAT CAC ATC GCA GAA GCG CTG GTG CGC TGG ATG GCA CCA ATC CTC TCC      2304
Tyr His Ile Ala Glu Ala Leu Val Arg Trp Met Ala Pro Ile Leu Ser
        755                 760                 765

TTC ACC GCT GAT GAA GTG TGG GGC TAC CTG CCG GGC GAA CGT GAA AAA      2352
Phe Thr Ala Asp Glu Val Trp Gly Tyr Leu Pro Gly Glu Arg Glu Lys
    770                 775                 780
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TAC GTC TTG ACC GGT GAG TGG TAC GAA GGC CTG TTT GGC CTG GCA GAC      2400
Tyr Val Leu Thr Gly Glu Trp Tyr Glu Gly Leu Phe Gly Leu Ala Asp
785                 790                 795                 800

AGT GAA GCG ATG AAC GAT GCG TTC TGG GAC GAG CTG TTG AAA GTG CGT      2448
Ser Glu Ala Met Asn Asp Ala Phe Trp Asp Glu Leu Leu Lys Val Arg
                805                 810                 815

GGC GAA GTG AAC AAA GTC ATT GAG CAA GCG CGT GCC GAC AAC AAA GTG      2496
Gly Glu Val Asn Lys Val Ile Glu Gln Ala Arg Ala Asp Asn Lys Val
            820                 825                 830

GGT GGC TCG CTG GAA GCG GCA GTA ACC TTG TAT GCA GAA CCG GAA CTG      2544
Gly Gly Ser Leu Glu Ala Ala Val Thr Leu Tyr Ala Glu Pro Glu Leu
        835                 840                 845

TCG GCG AAA CTG ACC GCG CTG GGC GAT GAA TTA CGA TTT GTC CTG TTG      2592
Ser Ala Lys Leu Thr Ala Leu Gly Asp Glu Leu Arg Phe Val Leu Leu
    850                 855                 860

ACC TCC GAC CGG CGC TAC GTT GCA GAC TAT AAC GAC GCA CCT GCT GAT      2640
Thr Ser Asp Arg Arg Tyr Val Ala Asp Tyr Asn Asp Ala Pro Ala Asp
865                 870                 875                 880

GCT CAG CAG AGC GAA GTA CTC AAA GGG CTG AAA GTC GCG TTG AGT AAA      2688
Ala Gln Gln Ser Glu Val Leu Lys Gly Leu Lys Val Ala Leu Ser Lys
                885                 890                 895

GCC GAA GGT GAG AAG TGC CCA CGC TGC TGG CAC TAC ACC CAG GAT GTC      2736
Ala Glu Gly Glu Lys Cys Pro Arg Cys Trp His Tyr Thr Gln Asp Val
            900                 905                 910

GGC AAG GTG GCG GAA CAC GCA GAA ATC TGC GGC CGC TGT GTC AGC AAC      2784
Gly Lys Val Ala Glu His Ala Glu Ile Cys Gly Arg Cys Val Ser Asn
        915                 920                 925

GTC GCC GGT GAC GGT GAA AAA CGT AAG TTT GCC TGA                      2820
Val Ala Gly Asp Gly Glu Lys Arg Lys Phe Ala
    930                 935                 940
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(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 939 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

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Met Ser Asp Tyr Lys Ser Thr Leu Asn Leu Pro Glu Thr Gly Phe Pro
  1               5                  10                  15

Met Arg Gly Asp Leu Ala Lys Arg Glu Pro Gly Met Leu Ala Arg Trp
            20                  25                  30

Thr Asp Asp Asp Leu Tyr Gly Ile Ile Arg Ala Ala Lys Lys Gly Lys
        35                  40                  45
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Lys Thr Phe Ile Leu His Asp Gly Pro Pro Tyr Ala Asn Gly Ser Ile
 50                  55                  60

His Ile Gly His Ser Val Asn Lys Ile Leu Lys Asp Ile Ile Val Lys
65                  70                  75                  80

Ser Lys Gly Leu Ser Gly Tyr Asp Ser Pro Tyr Val Pro Gly Trp Asp
                85                  90                  95

Cys His Gly Leu Pro Ile Glu Leu Lys Val Glu Gln Glu Tyr Gly Lys
            100                 105                 110

Pro Gly Glu Lys Phe Thr Ala Ala Glu Phe Arg Ala Lys Cys Arg Glu
        115                 120                 125

Tyr Ala Ala Thr Gln Val Asp Gly Gln Arg Lys Asp Phe Ile Arg Leu
    130                 135                 140

Gly Val Leu Gly Asp Trp Ser His Pro Tyr Leu Thr Met Asp Phe Lys
145                 150                 155                 160

Thr Glu Ala Asn Ile Ile Arg Ala Leu Gly Lys Ile Ile Gly Asn Gly
                165                 170                 175

His Leu His Lys Gly Ala Lys Pro Val His Trp Cys Val Asp Cys Arg
            180                 185                 190

Ser Ala Leu Ala Glu Ala Glu Val Glu Tyr Tyr Asp Lys Thr Ser Pro
        195                 200                 205

Ser Ile Asp Val Ala Phe Gln Ala Val Asp Gln Asp Ala Leu Lys Ala
    210                 215                 220

Lys Phe Ala Val Ser Asn Val Asn Gly Pro Ile Ser Leu Val Ile Trp
225                 230                 235                 240

Thr Thr Thr Pro Trp Thr Leu Pro Ala Asn Arg Ala Ile Ser Ile Ala
                245                 250                 255

Pro Asp Phe Asp Tyr Ala Leu Val Gln Ile Asp Gly Gln Ala Val Ile
            260                 265                 270

Leu Ala Lys Asp Leu Val Glu Ser Val Met Gln Arg Ile Gly Val Thr
        275                 280                 285

Asp Tyr Thr Ile Leu Gly Thr Val Lys Gly Ala Asp Val Glu Leu Leu
    290                 295                 300

Arg Phe Thr His Pro Phe Met Gly Phe Asp Val Pro Ala Ile Leu Gly
305                 310                 315                 320

Asp His Val Thr Leu Asp Ala Gly Thr Gly Ala Val His Thr Ala Pro
                325                 330                 335

Gly His Gly Pro Asp Asp Tyr Val Ile Gly Gln Lys Tyr Gly Leu Glu
            340                 345                 350

Thr Ala Asn Pro Val Gly Pro Asp Gly Thr Tyr Leu Pro Gly Thr Tyr
        355                 360                 365

Pro Thr Leu Asp Gly Val Asn Val Phe Lys Ala Asn Asp Ile Val Val
    370                 375                 380
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Ala Leu Leu Gln Glu Lys Gly Ala Leu Leu His Val Glu Lys Met Gln
385 390 395 400

His Ser Tyr Pro Cys Cys Trp Arg His Lys Thr Pro Ile Ile Phe Arg
405 410 415

Ala Thr Pro Gln Trp Phe Val Ser Met Asp Gln Lys Gly Leu Arg Ala
420 425 430

Gln Ser Leu Lys Glu Ile Lys Gly Val Gln Trp Ile Pro Asp Trp Gly
435 440 445

Gln Ala Arg Ile Glu Ser Met Val Ala Asn Arg Pro Asp Trp Cys Ile
450 455 460

Ser Arg Gln Arg Thr Trp Gly Val Pro Met Ser Leu Phe Val His Lys
465 470 475 480

Asp Thr Glu Glu Leu His Pro Arg Thr Leu Glu Leu Met Glu Glu Val
485 490 495

Ala Lys Arg Val Glu Val Asp Gly Ile Gln Ala Trp Trp Asp Leu Asp
500 505 510

Ala Lys Glu Ile Leu Gly Asp Glu Ala Asp Gln Tyr Val Lys Val Pro
515 520 525

Asp Thr Leu Asp Val Trp Phe Asp Ser Gly Ser Thr His Ser Ser Val
530 535 540

Val Asp Val Arg Pro Glu Phe Ala Gly His Ala Ala Asp Met Tyr Leu
545 550 555 560

Glu Gly Ser Asp Gln His Arg Gly Trp Phe Met Ser Ser Leu Met Ile
565 570 575

Ser Thr Ala Met Lys Gly Lys Ala Pro Tyr Arg Gln Val Leu Thr His
580 585 590

Gly Phe Thr Val Asp Gly Gln Gly Arg Lys Met Ser Lys Ser Ile Gly
595 600 605

Asn Thr Val Ser Pro Gln Asp Val Met Asn Lys Leu Gly Ala Asp Ile
610 615 620

Leu Arg Leu Trp Val Ala Ser Thr Asp Tyr Thr Gly Gln Met Ala Val
625 630 635 640

Ser Asp Glu Ile Leu Lys Arg Ala Ala Asp Ser Tyr Arg Arg Ile Arg
645 650 655

Asn Thr Ala Arg Phe Leu Leu Ala Asn Leu Asn Gly Phe Asp Pro Ala
660 665 670

Lys Asp Met Val Lys Pro Glu Glu Met Val Val Leu Asp Arg Trp Ala
675 680 685

Val Gly Cys Ala Lys Ala Ala Gln Glu Asp Ile Leu Lys Ala Tyr Glu
690 695 700

Ala Tyr Asp Phe His Glu Val Val Gln Arg Leu Met Arg Phe Cys Ser
705 710 715 720

Val Glu Met Gly Ser Phe Tyr Leu Asp Ile Ile Lys Asp Arg Gln Tyr
725 730 735

Thr Pro Lys Arg Thr Val Trp Ala Arg Arg Ser Cys Gln Thr Ala Leu
740 745 750

Tyr His Ile Ala Glu Ala Leu Val Arg Trp Met Ala Pro Ile Leu Ser
755 760 765

Phe Thr Ala Asp Glu Val Trp Gly Tyr Leu Pro Gly Glu Arg Glu Lys
770 775 780

Tyr Val Leu Thr Gly Glu Trp Tyr Glu Gly Leu Phe Gly Leu Ala Asp
785 790 795 800

Ser Glu Ala Met Asn Asp Ala Phe Trp Asp Glu Leu Leu Lys Val Arg
805 810 815

Gly Glu Val Asn Lys Val Ile Glu Gln Ala Arg Ala Asp Asn Lys Val
820 825 830

Gly Gly Ser Leu Glu Ala Ala Val Thr Leu Tyr Ala Glu Pro Glu Leu
835 840 845

Ser Ala Lys Leu Thr Ala Leu Gly Asp Glu Leu Arg Phe Val Leu Leu
850 855 860

Thr Ser Asp Arg Arg Tyr Val Ala Asp Tyr Asn Asp Ala Pro Ala Asp
865 870 875 880

Ala Gln Gln Ser Glu Val Leu Lys Gly Leu Lys Val Ala Leu Ser Lys
885 890 895

Ala Glu Gly Glu Lys Cys Pro Arg Cys Trp His Tyr Thr Gln Asp Val
900 905 910

Gly Lys Val Ala Glu His Ala Glu Ile Cys Gly Arg Cys Val Ser Asn
915 920 925

Val Ala Gly Asp Gly Glu Lys Arg Lys Phe Ala
930 935

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2811 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..2811

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

ATG AGT GAC TAT AAA TCA ACC CTG AAT TTG CCG GAA ACA GGG TTC CCG 48
Met Ser Asp Tyr Lys Ser Thr Leu Asn Leu Pro Glu Thr Gly Phe Pro
1 5 10 15

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ATG CGT GGC GAT CTC GCC AAG CGC GAA CCC GGA ATG CTG GCG CGT TGG          96
Met Arg Gly Asp Leu Ala Lys Arg Glu Pro Gly Met Leu Ala Arg Trp
                20                  25                  30

ACT GAT GAT GAT CTG TAC GGC ATC ATC CGT GCG GCT AAA AAA GGC AAA         144
Thr Asp Asp Asp Leu Tyr Gly Ile Ile Arg Ala Ala Lys Lys Gly Lys
            35                  40                  45

AAA ACC TTC ATT CTG CAT GAT GGC CCT CCT TAT GCG AAT GGC AGC ATT         192
Lys Thr Phe Ile Leu His Asp Gly Pro Pro Tyr Ala Asn Gly Ser Ile
        50                  55                  60

CAT ATT GGT CAC TCG GTT AAC AAG ATT CTG AAA GAC ATT ATC GTG AAG         240
His Ile Gly His Ser Val Asn Lys Ile Leu Lys Asp Ile Ile Val Lys
 65                  70                  75                  80

TCC AAA GGG CTT TCC GGT TAT GAC TCG CCG TAT GTG CCT GGC TGG GAC         288
Ser Lys Gly Leu Ser Gly Tyr Asp Ser Pro Tyr Val Pro Gly Trp Asp
                85                  90                  95

TGC CAC GGT CTG CCG ATC GAG CTG AAA GTC GAG CAA GAA TAC GGT AAG         336
Cys His Gly Leu Pro Ile Glu Leu Lys Val Glu Gln Glu Tyr Gly Lys
            100                 105                 110

CCG GGT GAG AAA TTC ACC GCC GCC GAG TTC CGC GCC AAG TGC CGC GAA         384
Pro Gly Glu Lys Phe Thr Ala Ala Glu Phe Arg Ala Lys Cys Arg Glu
        115                 120                 125

TAC GCG GCG ACC CAG GTT GAC GGT CAA CGC AAA GAC TTT ATC CGT CTG         432
Tyr Ala Ala Thr Gln Val Asp Gly Gln Arg Lys Asp Phe Ile Arg Leu
    130                 135                 140

GGC GTG CTG GGC GAC TGG TCG CAC CCG TAC CTG ACC ATG GGA CTT CAA         480
Gly Val Leu Gly Asp Trp Ser His Pro Tyr Leu Thr Met Gly Leu Gln
145                 150                 155                 160

AAC TGG AAG GCC AAC ATC ATC CGC GCG CTG GGC AAA ATC ATC GGC AAC         528
Asn Trp Lys Ala Asn Ile Ile Arg Ala Leu Gly Lys Ile Ile Gly Asn
                165                 170                 175

GGT CAC CTG CAC AAA GGC GCG AAG CCA GTT CAC TGG TGC GTT GAC TGC         576
Gly His Leu His Lys Gly Ala Lys Pro Val His Trp Cys Val Asp Cys
            180                 185                 190

CGT TCT GCG CTG GCG AAG CGG AAG TTG AGT ATT ACG ACA AAA CTT CTC         624
Arg Ser Ala Leu Ala Lys Arg Lys Leu Ser Ile Thr Thr Lys Leu Leu
        195                 200                 205

CGT CCA TCG ACG TTG CTT TCC AGG GCA GTC GAT CAG GAT GCA CTG AAA         672
Arg Pro Ser Thr Leu Leu Ser Arg Ala Val Asp Gln Asp Ala Leu Lys
    210                 215                 220

GCA AAA TTT GCC GTA AGC AAC GTT AAC GGC CCA ATC TCG CTG GTA ATC         720
Ala Lys Phe Ala Val Ser Asn Val Asn Gly Pro Ile Ser Leu Val Ile
225                 230                 235                 240

TGG ACC ACC ACG CCG TGG ACT CTG CCT GCC AAC CGC GCA ATC TCT ATT         768
Trp Thr Thr Thr Pro Trp Thr Leu Pro Ala Asn Arg Ala Ile Ser Ile
                245                 250                 255
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GCA CCA GAT TTC GAC TAT GCG CTG GTG CAG ATC GAC GGT CAG GCC GTG          816
Ala Pro Asp Phe Asp Tyr Ala Leu Val Gln Ile Asp Gly Gln Ala Val
            260                 265                 270

ATT CTG GCG AAA GAT CTG GTT GAA AGC GTA ATG CAG CGT ATC GGC GTG          864
Ile Leu Ala Lys Asp Leu Val Glu Ser Val Met Gln Arg Ile Gly Val
        275                 280                 285

ACC GAT TCT CGG CAC GGT AAA AGG TGC GGA GCT GGA GCC GCT GCG TTT          912
Thr Asp Ser Arg His Gly Lys Arg Cys Gly Ala Gly Ala Ala Ala Phe
    290                 295                 300

ACC CAT CCG TTT ATG GGC TTC GAC GTT CCG GCA ATC CTC GGC GAT CAC          960
Thr His Pro Phe Met Gly Phe Asp Val Pro Ala Ile Leu Gly Asp His
305                 310                 315                 320

GTT ACC CTG GAT GCC GGT ACC GGT GCC GTT CAC ACC GCG CCT GGC CAC         1008
Val Thr Leu Asp Ala Gly Thr Gly Ala Val His Thr Ala Pro Gly His
                325                 330                 335

GGC CCG GAC GAC TAT GTG ATC GGT CAG AAA TAC GGC CTG GAA ACC GCT         1056
Gly Pro Asp Asp Tyr Val Ile Gly Gln Lys Tyr Gly Leu Glu Thr Ala
            340                 345                 350

AAC CCG GTT GGC CCG GAC GGC ACT TAT CTG CCG GGC ACT TAT CCG ACG         1104
Asn Pro Val Gly Pro Asp Gly Thr Tyr Leu Pro Gly Thr Tyr Pro Thr
        355                 360                 365

CTG GAT GGC GTG AAC GTC TTC AAA GCG AAC GAC ATC GTC GCT GCG CTG         1152
Leu Asp Gly Val Asn Val Phe Lys Ala Asn Asp Ile Val Ala Ala Leu
    370                 375                 380

CTG CAG GAA AAA GGC GCG CTG CTG CAC GTT GAG AAA ATG CAG CAC AGC         1200
Leu Gln Glu Lys Gly Ala Leu Leu His Val Glu Lys Met Gln His Ser
385                 390                 395                 400

TAT CCG TGC TGC TGG CGT CAC AAA ACG CCG ATC ATC TTC CGC GCG ACG         1248
Tyr Pro Cys Cys Trp Arg His Lys Thr Pro Ile Ile Phe Arg Ala Thr
                405                 410                 415

CCG CAG TGG TTC GTC AGC ATG GAT CAG AAA GGT CTG CGT GCG CAG TCA         1296
Pro Gln Trp Phe Val Ser Met Asp Gln Lys Gly Leu Arg Ala Gln Ser
            420                 425                 430

CTG AAA GAG ATC AAA GGC GTG CAG TGG ATC CCG GAC TGG GGC CAG GCG         1344
Leu Lys Glu Ile Lys Gly Val Gln Trp Ile Pro Asp Trp Gly Gln Ala
        435                 440                 445

CGT ATC GAG TCG ATG GTT GCT AAC CGT CCT GAC TGG TGT ATC TCC CGT         1392
Arg Ile Glu Ser Met Val Ala Asn Arg Pro Asp Trp Cys Ile Ser Arg
    450                 455                 460

CAG CGC ACC TGG GGT GTA CCG ATG TCA CTG TTC GTG CAC AAA GAC ACG         1440
Gln Arg Thr Trp Gly Val Pro Met Ser Leu Phe Val His Lys Asp Thr
465                 470                 475                 480

GAA GAG CTG CAT CCG CGT ACC CTT GAA CTG ATG GAA GAA GTG GCA AAA         1488
Glu Glu Leu His Pro Arg Thr Leu Glu Leu Met Glu Glu Val Ala Lys
                485                 490                 495
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CGC GTT GAA GTC GAT GGC ATC CAG GCG TGG TGG GAT CTC GAT GCG AAA      1536
Arg Val Glu Val Asp Gly Ile Gln Ala Trp Trp Asp Leu Asp Ala Lys
            500                 505                 510

GAG ATC CTC GGC GAC GAA GCT GAT CAG TAC GTG AAA GTG CCG GAC ACA      1584
Glu Ile Leu Gly Asp Glu Ala Asp Gln Tyr Val Lys Val Pro Asp Thr
        515                 520                 525

TTG GAT GTA TGG TTT GAC TCC GGA TCT ACC CAC TCT TCT GTT GTT GAC      1632
Leu Asp Val Trp Phe Asp Ser Gly Ser Thr His Ser Ser Val Val Asp
    530                 535                 540

GTG CGT CCG GAA TTT GCC GGT CAC GCA GCG GAC ATG TAT CTG GAA GGT      1680
Val Arg Pro Glu Phe Ala Gly His Ala Ala Asp Met Tyr Leu Glu Gly
545                 550                 555                 560

TCT GAC CAA CAC CGC GGC TGG TTC ATG TCT TCC CTA ATG ATC TCC ACC      1728
Ser Asp Gln His Arg Gly Trp Phe Met Ser Ser Leu Met Ile Ser Thr
                565                 570                 575

GCG ATG AAG GGT AAA GCG CCG TAT CGT CAG GTA CTC ACC CAC GGC TTT      1776
Ala Met Lys Gly Lys Ala Pro Tyr Arg Gln Val Leu Thr His Gly Phe
            580                 585                 590

ACC GTG GAT GGT CAG GGC CGC AAG ATG TCT AAA TCC ATC GGC AAT ACC      1824
Thr Val Asp Gly Gln Gly Arg Lys Met Ser Lys Ser Ile Gly Asn Thr
        595                 600                 605

GTT TCG CCG CAG GAT GTG ATG AAC AAA CTG GGC GCG GAT ATT CTG CGT      1872
Val Ser Pro Gln Asp Val Met Asn Lys Leu Gly Ala Asp Ile Leu Arg
    610                 615                 620

CTG TGG GTG GCA TCA ACC GAC TAC ACC GGT GAA ATG GCC GTT TCT GAC      1920
Leu Trp Val Ala Ser Thr Asp Tyr Thr Gly Glu Met Ala Val Ser Asp
625                 630                 635                 640

GAG ATC CTG AAA CGT GCT GCC GAT ACG TAT CGT CGT ATC CGT AAC ACC      1968
Glu Ile Leu Lys Arg Ala Ala Asp Thr Tyr Arg Arg Ile Arg Asn Thr
                645                 650                 655

GCG CGC TTC CTG CTG GCA AAC CTG AAC GGT TTT GAT CCA GCA AAA GAT      2016
Ala Arg Phe Leu Leu Ala Asn Leu Asn Gly Phe Asp Pro Ala Lys Asp
            660                 665                 670

ATG GTG AAA CGG AGA GAG ATG GTG GTA CTG GAT CGC TGG GCC GTA GTT      2064
Met Val Lys Arg Arg Glu Met Val Val Leu Asp Arg Trp Ala Val Val
        675                 680                 685

GTG CGA AAG CGG CAC AGG AAG ACA TCC TCA AGG CGT ACG AAG CAT ACG      2112
Val Arg Lys Arg His Arg Lys Thr Ser Ser Arg Arg Thr Lys His Thr
    690                 695                 700

ATT TCC ACG AAG TGG TAC AAG CGT CTG ATG CGC TTC TGC TCC GTT GAG      2160
Ile Ser Thr Lys Trp Tyr Lys Arg Leu Met Arg Phe Cys Ser Val Glu
705                 710                 715                 720

ATG GGT TCC TTC TAC CTC GAC ATC ATC AAA GAC CGT CAG TAC TAC GCC      2208
Met Gly Ser Phe Tyr Leu Asp Ile Ile Lys Asp Arg Gln Tyr Tyr Ala
                725                 730                 735
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AAA GGA CAC AGT GTG GCG CGT CGT AGC TGC CAG ACT GCG CTA TAT CAC      2256
Lys Gly His Ser Val Ala Arg Arg Ser Cys Gln Thr Ala Leu Tyr His
            740                 745                 750

ATC GCA GAA GCG CTG GTG CGC TGG ATG GCA CCA ATC CTC TCC TTC ACC      2304
Ile Ala Glu Ala Leu Val Arg Trp Met Ala Pro Ile Leu Ser Phe Thr
        755                 760                 765

GCT GAT GAA GTG TGG GGC TAC CTG CCG GGC GAA CGT GAA AAA TAC GTC      2352
Ala Asp Glu Val Trp Gly Tyr Leu Pro Gly Glu Arg Glu Lys Tyr Val
    770                 775                 780

TTC ACC GGT GAG TGG TAC GAA GGC CTG TTT GGC CTG GCA GAC AGT GAA      2400
Phe Thr Gly Glu Trp Tyr Glu Gly Leu Phe Gly Leu Ala Asp Ser Glu
785                 790                 795                 800

GCG ATG AAC GAT GCG TTC TGG GAC GAG CTG TTG AAA GTG CGT GGC GAA      2448
Ala Met Asn Asp Ala Phe Trp Asp Glu Leu Leu Lys Val Arg Gly Glu
                805                 810                 815

GTG AAC AAA GTC ATT GAG CAA GCG CGT GCC GAC AAG AAA GTG GGT GGC      2496
Val Asn Lys Val Ile Glu Gln Ala Arg Ala Asp Lys Lys Val Gly Gly
            820                 825                 830

TCG CTG GAA GCG GCA GTA ACC TTG TAT GCA GAA CCG GAA CTG TCG GCG      2544
Ser Leu Glu Ala Ala Val Thr Leu Tyr Ala Glu Pro Glu Leu Ser Ala
        835                 840                 845

AAA CTG ACC GCG CTG GGC GAT GAA TTA CGA TTT GTC CTG TTG ACC TCC      2592
Lys Leu Thr Ala Leu Gly Asp Glu Leu Arg Phe Val Leu Leu Thr Ser
    850                 855                 860

GGC GCT ACC GTT GCA GAC TAT AAC GAC GCA CCT GCT GAT GCT CAG CAG      2640
Gly Ala Thr Val Ala Asp Tyr Asn Asp Ala Pro Ala Asp Ala Gln Gln
865                 870                 875                 880

AGC GAA GTA CTC AAA GGG CTG AAA GTC GCG TTG AGT AAA GCC GAA GGT      2688
Ser Glu Val Leu Lys Gly Leu Lys Val Ala Leu Ser Lys Ala Glu Gly
                885                 890                 895

GAG AAG TGC CCA CGC TGC TGG CAC TAC ACC CAG GAT GTC GGC AAG GTG      2736
Glu Lys Cys Pro Arg Cys Trp His Tyr Thr Gln Asp Val Gly Lys Val
            900                 905                 910

GCG GAA CAC GCA GAA ATC TGC GGC CGC TGT GTC AGC AAC GTC GCC GGT      2784
Ala Glu His Ala Glu Ile Cys Gly Arg Cys Val Ser Asn Val Ala Gly
        915                 920                 925

GAC GGT GAA AAA CGT AAG TTT GCC TGA                                  2811
Asp Gly Glu Lys Arg Lys Phe Ala
    930                 935
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 936 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Met Ser Asp Tyr Lys Ser Thr Leu Asn Leu Pro Glu Thr Gly Phe Pro
1 5 10 15

Met Arg Gly Asp Leu Ala Lys Arg Glu Pro Gly Met Leu Ala Arg Trp
20 25 30

Thr Asp Asp Asp Leu Tyr Gly Ile Ile Arg Ala Ala Lys Lys Gly Lys
35 40 45

Lys Thr Phe Ile Leu His Asp Gly Pro Pro Tyr Ala Asn Gly Ser Ile
50 55 60

His Ile Gly His Ser Val Asn Lys Ile Leu Lys Asp Ile Ile Val Lys
65 70 75 80

Ser Lys Gly Leu Ser Gly Tyr Asp Ser Pro Tyr Val Pro Gly Trp Asp
85 90 95

Cys His Gly Leu Pro Ile Glu Leu Lys Val Glu Gln Glu Tyr Gly Lys
100 105 110

Pro Gly Glu Lys Phe Thr Ala Ala Glu Phe Arg Ala Lys Cys Arg Glu
115 120 125

Tyr Ala Ala Thr Gln Val Asp Gly Gln Arg Lys Asp Phe Ile Arg Leu
130 135 140

Gly Val Leu Gly Asp Trp Ser His Pro Tyr Leu Thr Met Gly Leu Gln
145 150 155 160

Asn Trp Lys Ala Asn Ile Ile Arg Ala Leu Gly Lys Ile Ile Gly Asn
165 170 175

Gly His Leu His Lys Gly Ala Lys Pro Val His Trp Cys Val Asp Cys
180 185 190

Arg Ser Ala Leu Ala Lys Arg Lys Leu Ser Ile Thr Thr Lys Leu Leu
195 200 205

Arg Pro Ser Thr Leu Leu Ser Arg Ala Val Asp Gln Asp Ala Leu Lys
210 215 220

Ala Lys Phe Ala Val Ser Asn Val Asn Gly Pro Ile Ser Leu Val Ile
225 230 235 240

Trp Thr Thr Thr Pro Trp Thr Leu Pro Ala Asn Arg Ala Ile Ser Ile
245 250 255

Ala Pro Asp Phe Asp Tyr Ala Leu Val Gln Ile Asp Gly Gln Ala Val
260 265 270

Ile Leu Ala Lys Asp Leu Val Glu Ser Val Met Gln Arg Ile Gly Val
275 280 285

Thr Asp Ser Arg His Gly Lys Arg Cys Gly Ala Gly Ala Ala Ala Phe
290 295 300

Thr His Pro Phe Met Gly Phe Asp Val Pro Ala Ile Leu Gly Asp His
305 310 315 320

Val Thr Leu Asp Ala Gly Thr Gly Ala Val His Thr Ala Pro Gly His
325 330 335

Gly Pro Asp Asp Tyr Val Ile Gly Gln Lys Tyr Gly Leu Glu Thr Ala
340 345 350

Asn Pro Val Gly Pro Asp Gly Thr Tyr Leu Pro Gly Thr Tyr Pro Thr
355 360 365

Leu Asp Gly Val Asn Val Phe Lys Ala Asn Asp Ile Val Ala Ala Leu
370 375 380

Leu Gln Glu Lys Gly Ala Leu Leu His Val Glu Lys Met Gln His Ser
385 390 395 400

Tyr Pro Cys Cys Trp Arg His Lys Thr Pro Ile Ile Phe Arg Ala Thr
405 410 415

Pro Gln Trp Phe Val Ser Met Asp Gln Lys Gly Leu Arg Ala Gln Ser
420 425 430

Leu Lys Glu Ile Lys Gly Val Gln Trp Ile Pro Asp Trp Gly Gln Ala
435 440 445

Arg Ile Glu Ser Met Val Ala Asn Arg Pro Asp Trp Cys Ile Ser Arg
450 455 460

Gln Arg Thr Trp Gly Val Pro Met Ser Leu Phe Val His Lys Asp Thr
465 470 475 480

Glu Glu Leu His Pro Arg Thr Leu Glu Leu Met Glu Glu Val Ala Lys
485 490 495

Arg Val Glu Val Asp Gly Ile Gln Ala Trp Trp Asp Leu Asp Ala Lys
500 505 510

Glu Ile Leu Gly Asp Glu Ala Asp Gln Tyr Val Lys Val Pro Asp Thr
515 520 525

Leu Asp Val Trp Phe Asp Ser Gly Ser Thr His Ser Ser Val Val Asp
530 535 540

Val Arg Pro Glu Phe Ala Gly His Ala Ala Asp Met Tyr Leu Glu Gly
545 550 555 560

Ser Asp Gln His Arg Gly Trp Phe Met Ser Ser Leu Met Ile Ser Thr
565 570 575

Ala Met Lys Gly Lys Ala Pro Tyr Arg Gln Val Leu Thr His Gly Phe
580 585 590

Thr Val Asp Gly Gln Gly Arg Lys Met Ser Lys Ser Ile Gly Asn Thr
595 600 605

Val Ser Pro Gln Asp Val Met Asn Lys Leu Gly Ala Asp Ile Leu Arg
610 615 620

Leu Trp Val Ala Ser Thr Asp Tyr Thr Gly Glu Met Ala Val Ser Asp
625 630 635 640

Glu Ile Leu Lys Arg Ala Ala Asp Thr Tyr Arg Arg Ile Arg Asn Thr
645 650 655

Ala Arg Phe Leu Leu Ala Asn Leu Asn Gly Phe Asp Pro Ala Lys Asp
660 665 670

Met Val Lys Arg Arg Glu Met Val Val Leu Asp Arg Trp Ala Val Val
675 680 685

Val Arg Lys Arg His Arg Lys Thr Ser Ser Arg Arg Thr Lys His Thr
690 695 700

Ile Ser Thr Lys Trp Tyr Lys Arg Leu Met Arg Phe Cys Ser Val Glu
705 710 715 720

Met Gly Ser Phe Tyr Leu Asp Ile Ile Lys Asp Arg Gln Tyr Tyr Ala
725 730 735

Lys Gly His Ser Val Ala Arg Arg Ser Cys Gln Thr Ala Leu Tyr His
740 745 750

Ile Ala Glu Ala Leu Val Arg Trp Met Ala Pro Ile Leu Ser Phe Thr
755 760 765

Ala Asp Glu Val Trp Gly Tyr Leu Pro Gly Glu Arg Glu Lys Tyr Val
770 775 780

Phe Thr Gly Glu Trp Tyr Glu Gly Leu Phe Gly Leu Ala Asp Ser Glu
785 790 795 800

Ala Met Asn Asp Ala Phe Trp Asp Glu Leu Leu Lys Val Arg Gly Glu
805 810 815

Val Asn Lys Val Ile Glu Gln Ala Arg Ala Asp Lys Lys Val Gly Gly
820 825 830

Ser Leu Glu Ala Ala Val Thr Leu Tyr Ala Glu Pro Glu Leu Ser Ala
835 840 845

Lys Leu Thr Ala Leu Gly Asp Glu Leu Arg Phe Val Leu Leu Thr Ser
850 855 860

Gly Ala Thr Val Ala Asp Tyr Asn Asp Ala Pro Ala Asp Ala Gln Gln
865 870 875 880

Ser Glu Val Leu Lys Gly Leu Lys Val Ala Leu Ser Lys Ala Glu Gly
885 890 895

Glu Lys Cys Pro Arg Cys Trp His Tyr Thr Gln Asp Val Gly Lys Val
900 905 910

Ala Glu His Ala Glu Ile Cys Gly Arg Cys Val Ser Asn Val Ala Gly
915 920 925

Asp Gly Glu Lys Arg Lys Phe Ala
930 935

## CLAIMS

1. A method of inhibiting growth of a microbial cell, comprising introducing a homologous mutant aminoacyl-tRNA synthetase into said microbial cell, wherein said mutant synthetase is a catalytically inactive mutant capable of tRNA binding and inhibiting growth of said microbial cell.

2. The method of Claim 1 wherein the mutant aminoacyl-tRNA synthetase is a mutant isoleucyl-tRNA synthetase.

3. The method of Claim 2 wherein the mutant isoleucyl-tRNA synthetase has a mutation e.g., at a position corresponding to position 56, position 96 or position 570 of the wild-type *Escherichia coli* isoleucyl-tRNA synthetase.

4. The method of Claim 1 wherein the microbial cell is *Escherichia coli*.

5. A method of inhibiting growth of a microbial cell comprising the steps of:
   (a) introducing into said cell a nucleic acid which encodes a mutant aminoacyl-tRNA synthetase or portion thereof capable of specific binding of cognate tRNA; and
   (b) maintaining said cells under conditions whereby the encoded mutant aminoacyl-tRNA synthetase is expressed in amounts sufficient to selectively inhibit the growth of said microbial cell.

6. The method of Claim 5, wherein the mutant aminoacyl-tRNA synthetase is derived from the microbial cell.

7. The method of Claim 5, wherein the mutant aminoacyl-tRNA synthetase is defective in aminoacylation.

8. Use of a tRNA-binding molecule for selectively inhibiting growth of a microbial cell pathogen present in a host of the pathogen, comprising introducing a tRNA-binding molecule into said microbial cell, wherein said tRNA-binding molecule is selectively toxic to the microbial cell present in the host.

9. Use of Claim 8, wherein the tRNA-binding molecule is an aminoacyl-tRNA synthetase or fragment thereof.

10. Use of Claim 9, wherein the aminoacyl-tRNA synthetase or fragment thereof is catalytically inactive.

11. Use of Claim 10, wherein the aminoacyl-tRNA synthetase or fragment thereof is derived from an aminoacyl-tRNA synthetase of the microbial pathogen.

12. Use of a nucleic acid molecule which encodes a mutant aminoacyl-tRNA synthetase for antimicrobial therapy against a microbial cell pathogen present in a host, wherein:

(a) said nucleic acid, which encodes a mutant aminoacyl-tRNA synthetase or portion thereof capable of specific binding of cognate tRNA, is introduced into the microbial cell present in said host; and

(b) said cells are maintained under conditions whereby the encoded mutant aminoacyl-tRNA synthetase is expressed in amounts sufficient to selectively inhibit the growth of said microbial cell.

13. Use of a nucleic acid molecule which encodes a mutant aminoacyl-tRNA synthetase for antimicrobial therapy in a host, wherein:
(a) said nucleic acid is introduced into cells of a host of a selected microbial pathogen; and
(b) said cells are maintained under conditions whereby the encoded mutant aminoacyl-tRNA synthetase is expressed in amounts sufficient to selectively inhibit the growth of the microbial pathogen present in said host.

14. Use of Claims 12 or 13, wherein the mutant aminoacyl-tRNA synthetase is derived from the selected microbial pathogen.

15. Use of Claims 12 or 13, wherein the mutant aminoacyl-tRNA synthetase is defective in aminoacylation.

16. An inactive mutant aminoacyl-tRNA synthetase or fragment thereof, capable of selective toxicity to a selected microbial pathogen present in a host of said pathogen.

17. The aminoacyl-tRNA synthetase of Claim 16 wherein the mutant aminoacyl-tRNA synthetase or fragment thereof is derived from an aminoacyl-tRNA synthetase of the microbial pathogen.

18. The aminoacyl-tRNA synthetase of Claim 17, wherein the mutant aminoacyl-tRNA synthetase is an isoleucyl-tRNA synthetase.

19. An isolated nucleic acid encoding the mutant aminoacyl-tRNA synthetase or fragment thereof of any one of Claims 16-18.

20. An inducible expression vector comprising the nucleic acid of Claim 19, wherein expression of said nucleic acid is under the control of an inducible promoter.

21. A mutant isoleucyl-tRNA synthetase or fragment thereof having a mutation selected from the group consisting of a mutation at a position corresponding to position 56, position 96 or position 570 of the wild-type *Escherichia coli* isoleucyl-tRNA synthetase.

22. A mutant isoleucyl-tRNA synthetase of Claim 21 wherein the mutation is an aspartic acid to alanine mutation at position 96.

23. An isolated nucleic acid encoding the mutant isoleucyl-tRNA synthetase or fragment thereof of any one of Claims 21-22.

24. An inducible expression vector comprising the nucleic acid of Claim 23, wherein expression of said nucleic acid is under the control of an inducible promoter.

25. Use of a tRNA binding molecule for selectively inhibiting the growth of a target cell introduced into a host, wherein the tRNA binding molecule, when introduced into said target cell, is capable of selective toxicity to the introduced target cells in the host.

26. Use of Claim 25 wherein the target cell introduced into the host is engineered to contain a tRNA whose function is essential to viability of the cell and selective toxicity of the tRNA binding molecule is mediated through interaction with the essential tRNA.

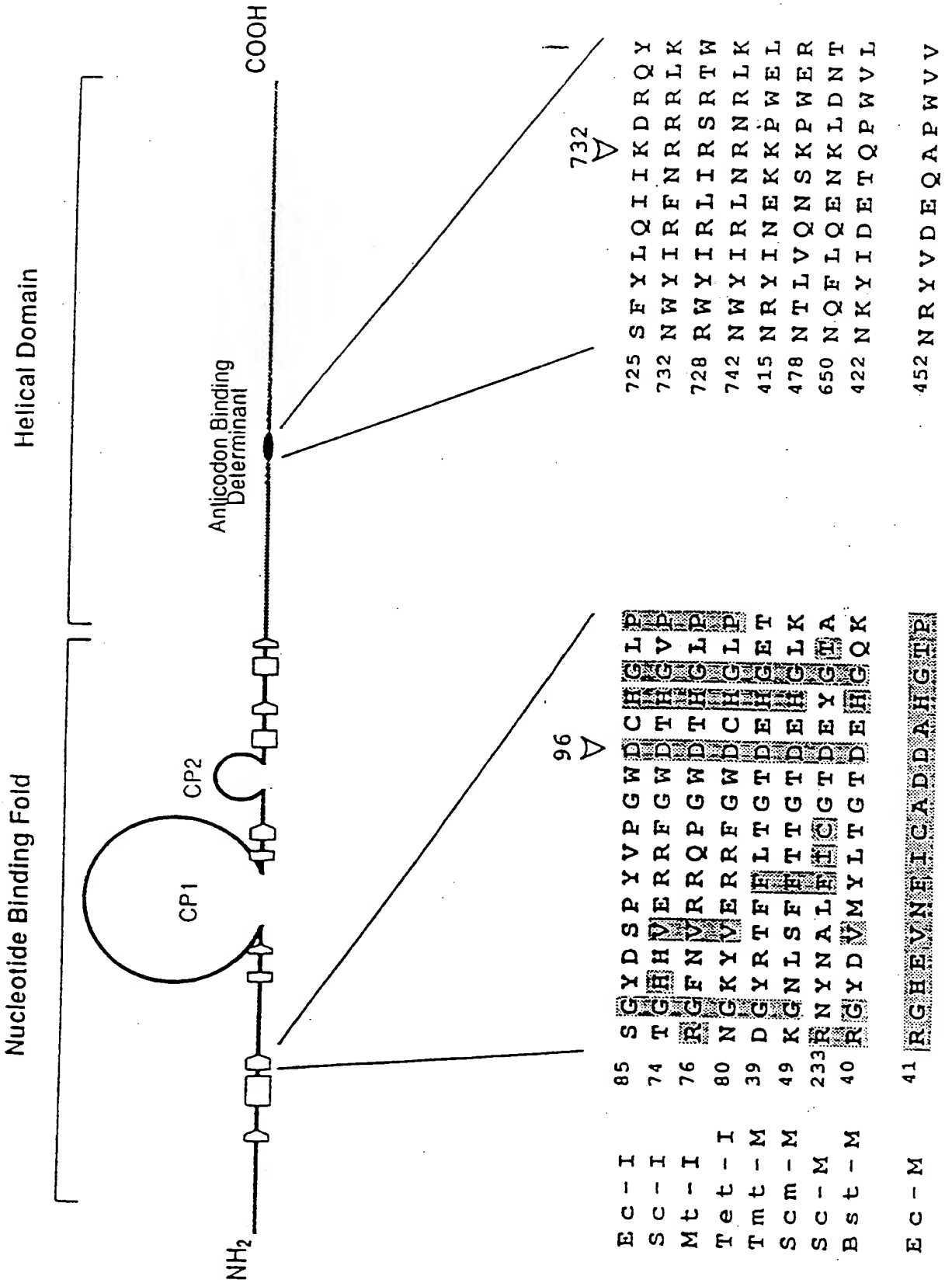

Helical Domain
Nucleotide Binding Fold
COOH
NH2
CP1
CP2
Anticodon Binding Determinant
96
732
1
Ec-I 85 S G Y D S P Y V P G W D C H G L P
Sc-I 74 T G H V E R R F G W D T H G V P
Mt-I 76 R G F N V R R Q P G W D T H G L P
Tet-I 80 N G K Y V E R R F G W D C H G L P
Tmt-M 39 D G Y R T F F L T G T D E H G E T
Scm-M 49 K G N L S F H T T G T D E H G L K
Sc-M 233 R N Y N A L H C G T D E Y G H A
Bst-M 40 R G Y D V M Y L T G T D E H G Q K
Ec-M 41 R G H E V N F I C A D D A H G T P
725 S F Y L Q I I K D R Q Y
732 N W Y I R F N R R R L K
728 R W Y I R L I R S R T W
742 N W Y I R L N R N R L K
415 N R Y I N E K K P W E L
478 N T L V Q N S K P W E R
650 N Q F L Q E N K L D N T
422 N K Y I D E T Q P W V L
452 N R Y V D E Q A P W V V

FIG. 1

FIG. 2A

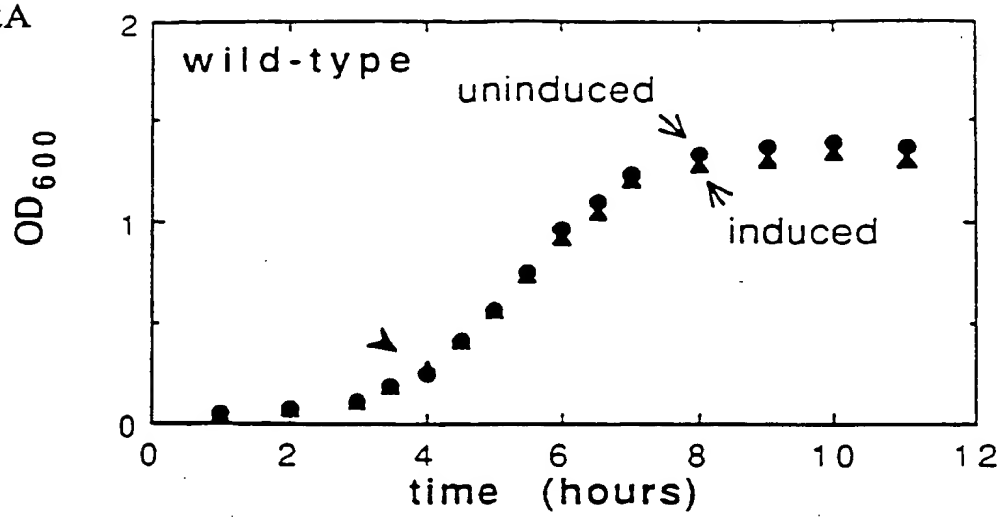



FIG. 2B

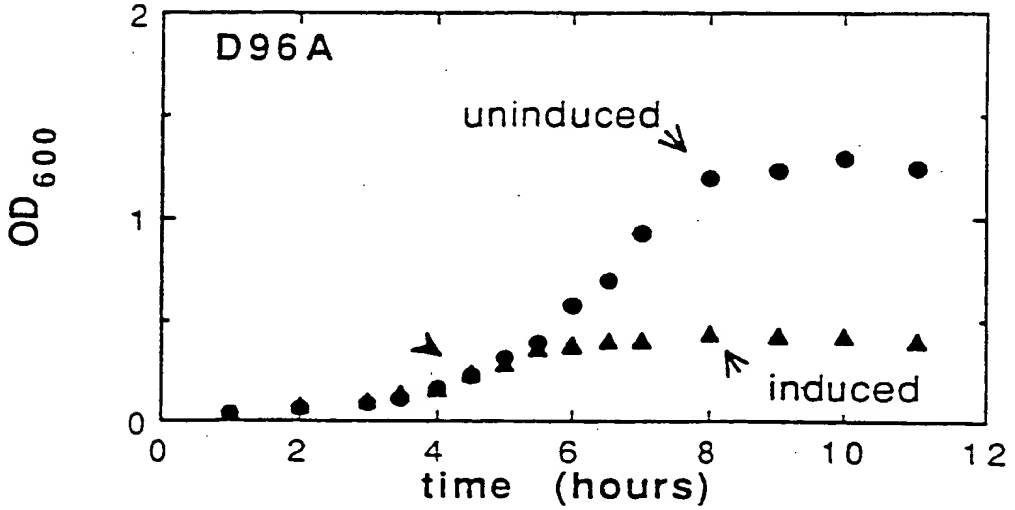
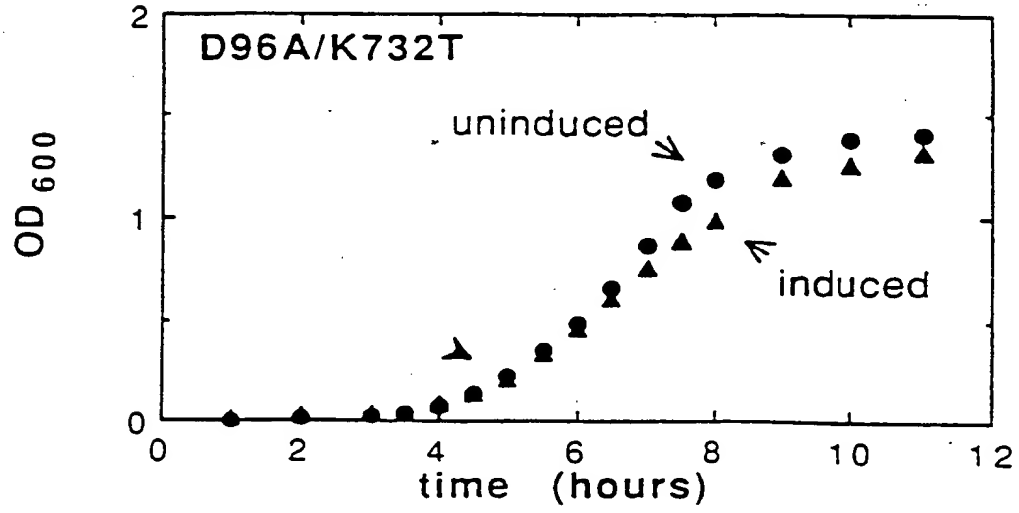



FIG. 2C



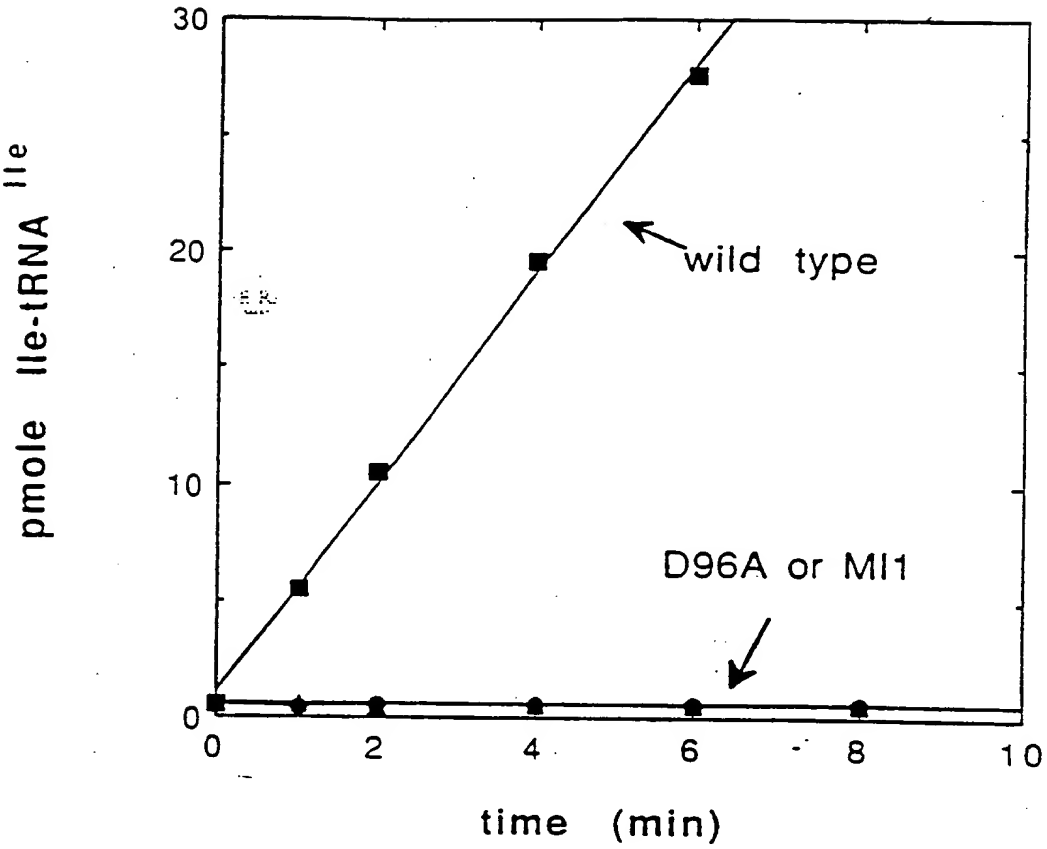

30
20
10
0
0
2
4
6
8
10
pmole Ile-tRNA Ile
time (min)
wild type
D96A or MI1

FIG. 3

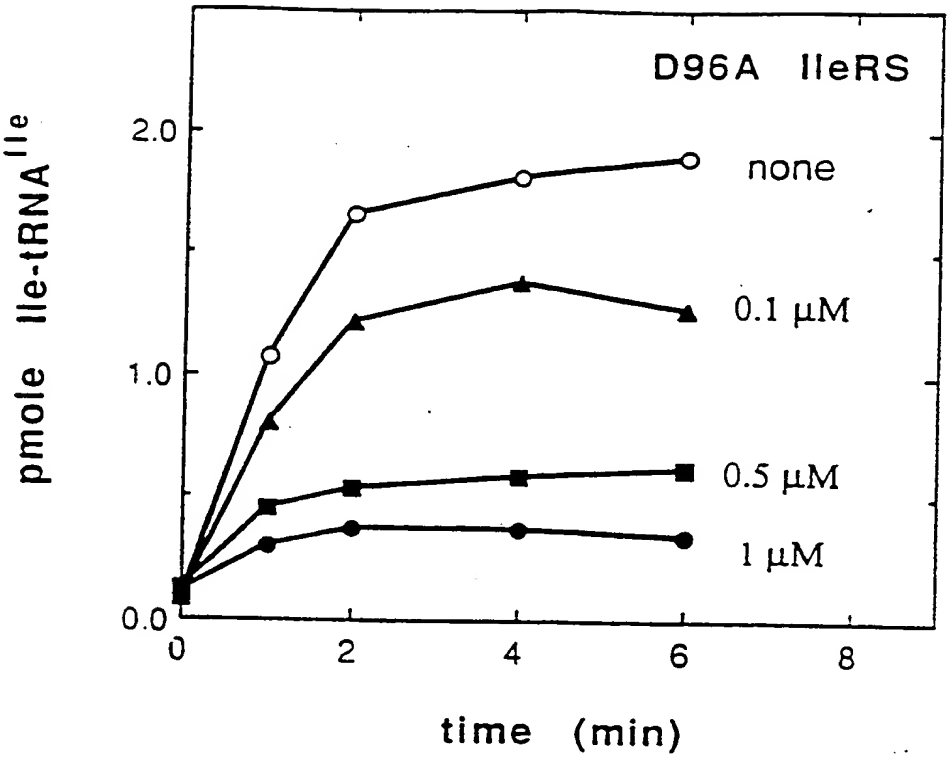

D96A IleRS
pmole Ile-tRNA$^{Ile}$
2.0
1.0
0.0
none
0.1 μM
0.5 μM
1 μM
0
2
4
6
8
time (min)

FIG. 4A

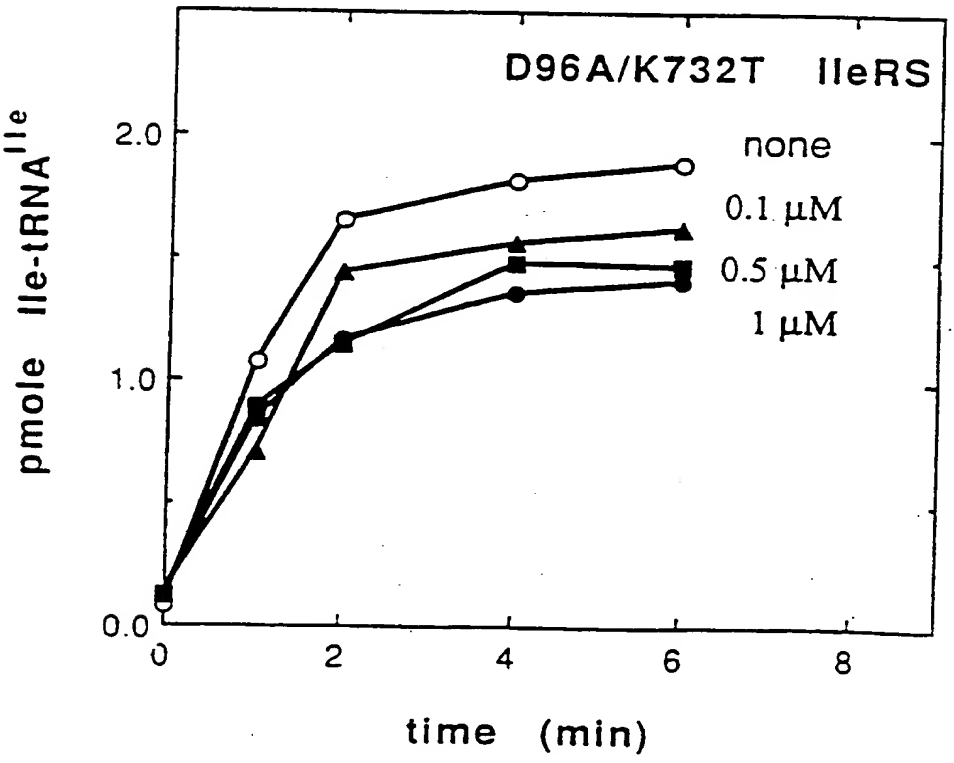

D96A/K732T IleRS
pmole Ile-tRNA$^{Ile}$
2.0
1.0
0.0
none
0.1 μM
0.5 μM
1 μM
0
2
4
6
8
time (min)

FIG. 4B

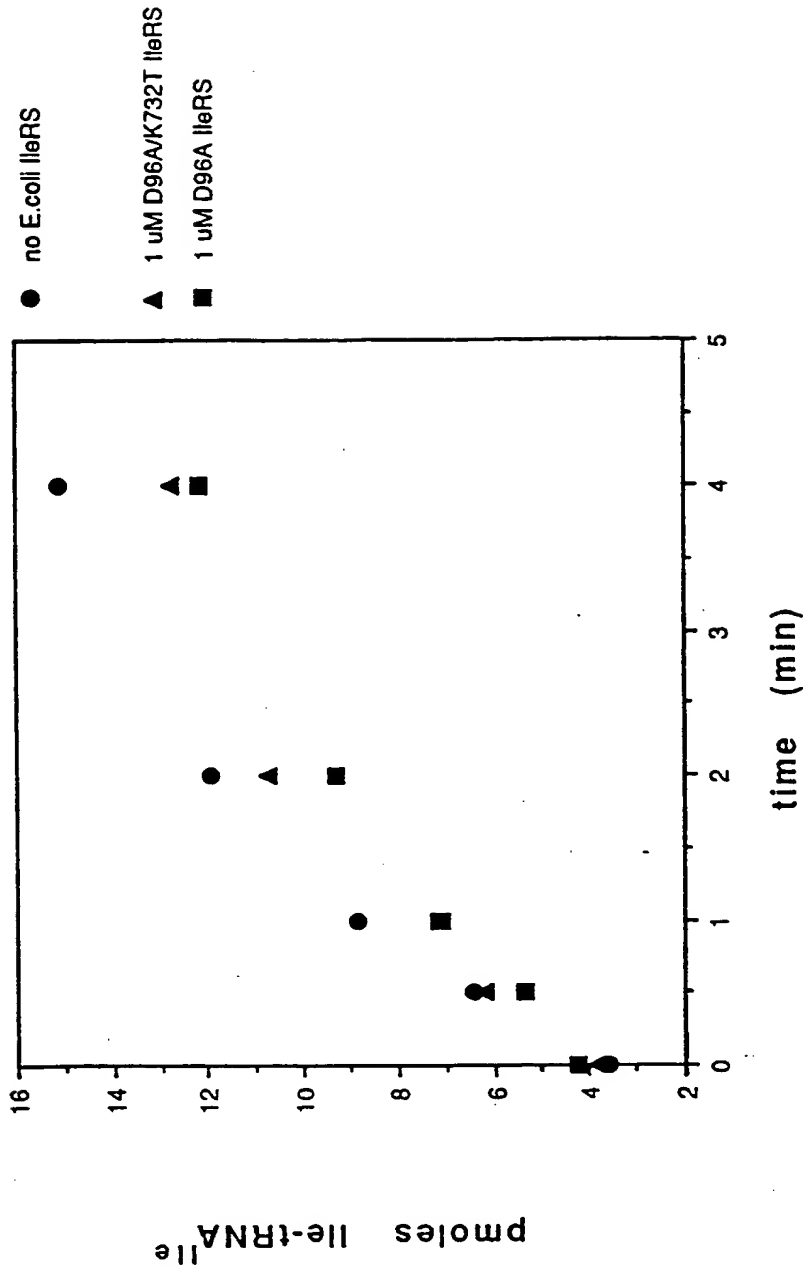

no E.coli IleRS
1 uM D96A/K732T IleRS
1 uM D96A IleRS
pmoles Ile-tRNA$^{Ile}$
time (min)
16
14
12
10
8
6
4
2
0
1
2
3
4
5

FIG. 5

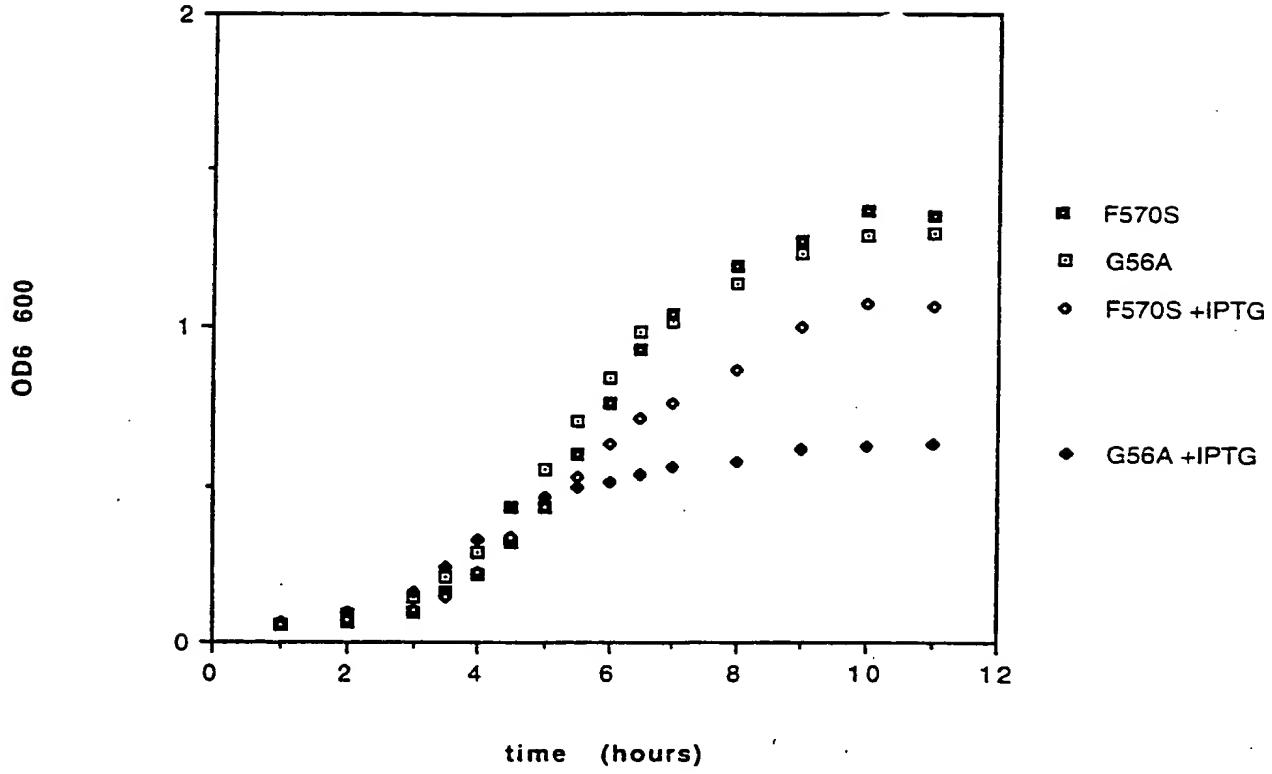

OD6 600
2
1
0
0
2
4
6
8
10
12
time (hours)
F570S
G56A
F570S +IPTG
G56A +IPTG

FIG. 6

# INTERNATIONAL SEARCH REPORT

Interna l Application No
PCT/US 94/05905

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/52 C12N9/00 A61K37/60

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)



C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | BIOCHEMISTRY<br>vol. 30 , 1991 , EASTON, PA US<br>pages 9569 - 9575<br>GHOSH, G. ET AL. 'Activation of methionine by Escherichia coli methionyl-tRNA-synthetase' cited in the application<br>see the whole document<br>--- | 1,4-17, 19,25,26 |
| Y | JOURNAL OF BACTERIOLOGY<br>vol. 172, no. 7 , July 1990 , WASHINGTON D.C., US<br>pages 3940 - 3945<br>BEDOUELLE, H. ET AL. 'Overproduction of tyrosyl-tRNA synthetase is toxic to Escherichia coli: a genetic analysis'<br>see the whole document, especially page 1944, right column, second paragraph<br>---<br>-/-- | 1,4-17, 19,25,26 |

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 12 September 1994 | -4 -10- 1994 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax (+31-70) 340-3016 | Andres, S |

Form PCT/ISA/210 (second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 94/05905

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | J BIOL CHEM 267 (22). 1992. 15563-15567<br>KIM, S. ET AL. 'FUNCTIONAL INDEPENDENCE OF MICROHELIX AMINOACYLATION FROM ANTICODON BINDING IN A CLASS I TRNA SYNTHETASE.'<br>cited in the application | |
| P,X | PROC. NATL. ACAD. SCI. U S A 90 (15), 1993, 6919-6923<br>SCHMIDT, E. ET AL. 'DOMINANT LETHALITY BY EXPRESSION OF A CATALYTICALLY INACTIVE CLASS I tRNA SYNTHETASE.'<br>see the whole document | 1-26 |

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05905

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 1-15,25-26,(as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the Human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:



1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.